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IDENTIFICATION AND CHARACTERISATION OF NEW APHID KILLING BACTERIA FOR USE AS BIOLOGICAL PEST CONTROL AGENTS

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Abstract

Insecticide resistance and on-going legislation changes on the use of insecticides are likely to reduce their availability for use in agriculture; hence there is an urgent need to develop effective biological controls against these plant pests. Aphids are major insect pests of the agricultural and horticultural sectors. Recent work screened a range of phylloplane-residing bacteria for their ability to kill aphids and used alternative insect targets to determine host specificity. Tests with five other aphid genera indicated the bacteria were also able to kill them. However, the bacteria were generally not effective against non-aphid species including Lepidoptera. I aimed to characterise potential aphid killing pathogens and investigate the killing mechanism. An artificial feeding system with a liquid diet was used to devise a high-throughput screening system to identify pathogenic bacteria against the Green Peach Aphid *Myzus persicae* ("wild type" insecticide susceptible clones plus insecticide resistant clones). Six bacterial strains were pathogenic to all insecticide susceptible and resistant clones although variation in susceptibility was observed. No single bacterial strain was identified that was consistently more toxic to insecticide resistance clones than susceptible clones, suggesting there was no penalty in resistant clones that makes such clones less fit to bacterial challenge. *Pseudomonas poae,* which was the most pathogenic to nearly all of aphid clones, was selected for further in-depth analyses. Plant colonisation assays showed that the bacterium could effectively grow and persist on three different plant species. Foliar spray of *P. poae* did not show any hypersensitive (HR) response and populations (log 5-6) remained stable over three weeks of infestation. Additionally, application of the bacterium to plants before aphid colonisation led to a 68 %, 57 %, 69 % reduction in aphid populations on pre-infested peppers, Arabidopsis and sugar beet plants, respectively. Olfactometer analysis showed that bacterial colonisation of leaves had a deterrent effect on aphids that was not evident for leaves or bacteria alone. Genome analysis of the bacterium revealed three different insecticidal toxins, stress response genes and other pathogenicity-related effector proteins which reflect potential toxicity towards aphids. RNA-Seq was used to examine changes in aphid and bacterial gene expression after 38 h of infection. The altered transcript profiles of the aphid revealed 193 differentially expressed genes and limited gene expression of lysosomal and detoxification genes. 1325 genes were differentially expressed in bacteria, which mainly includes iron acquisition and stress response genes, and putative toxins. Single and combinational deletion, and complementation, of different toxins was conducted. *In vitro* killing analysis indicated all toxins contributed to aphid killing, with a particularly strong effect seen for one, AprX. Together, these data are being used to understand the molecular basis of aphid mortality to bacterial infection with the aim of utilising them as effective biocontrol agents.

Declaration of original authorship

Declaration:

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

Deepa Paliwal.

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List of abbreviations

ANOVA	Analysis of variance
A. johnsonii	*Acinetobacter johnsonii*
B & H	Benjamini & hochberg
BHC	Beta-hexachlorocyclohexane
Bp	Base pair
Bt	*Bacillus thuringiensis*
cDNA	Complementary DNA
CDS	Coding DNA sequence
CFU mL^{-1}	Colony forming unit per mL
Cry	Insecticidal crystal protein
C. werkmanii	*Citrobacter werkmanii*
CT	Cycle threshold
DAPG	2, 4-diacetylphloroglucinol
DDT	Dichlorodiphenyltrichloroethane
DE	Differential expressed
DWA	Distilled water agar
E. albertii	*Escherichia albertii*
E. coli	*Escherichia coli*
E. fergusonii	*Escherichia fergusonii*
EC	Enzyme commission
ET	Ethylene
F	Forward primer
FC	Fold change
FDR	False discovery rate
Fit	For *P. Fluorescens* insecticidal toxin
FPKM	Fragments per kilobase of exon per million fragments mapped
g	Gram
gDNA	Genomic DNA
Gent	Gentamycin
GO	Gene ontology
H	Hours
HSD	Honest Significant Difference
IAA	Indole-3-acetate
IMD	Immune-deficiency
IPM	Integrated pest management
JA	Jasmonic acid
JAK/STAT	Janus kinase (JAK) and two Signal Transducer and Activator of Transcription
JNK	C-Jun N-terminal Kinase
Kan	Kanamycin
kb	Kilobase pair
Kdr	Knockdown resistance
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KMB	King's medium B

LB	Luria-Bertani
LC_{50}	Lethal concentration to kill 50 % population
MACE	Modified acetylcholinesterase
MB	Megabase pair
Mcf	Makes caterpillars' floppy
MCT	Multiple comparison test
mg	Milligram
µg	Microgram
MLST	Multilocus sequence typing
mRNA	Messenger RNA
ng	Nanogram
O/N	Overnight
OD	Optical density
Pae. glucanolyticus	*Paenibacillus glucanolyticus*
Pa. agglomerans	*Pantoea agglomerans*
PGRP	Peptidoglycan recognition proteins
Ph. luminescens	*Photorhabdus luminescens*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P. fluorescens	*Pseudomonas fluorescens*
P. jessenii	*Pseudomonas jessenii*
P. poae	*Pseudomonas poae*
P. rhizosphaerae	*Pseudomonas rhizosphaerae*
PLT	Pyoluteorin
qPCR	Quantitative/real-time PCR
QS	Quorum sensing
R	Reverse primer
R^2	Regression coefficient
RABT	Reference Annotation Based Transcript
RES	Resistant
RF	Resistance Factor
Rhs	Rearrangement hotspot
RIN	RNA integrity number
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
SA	Salicylic acid
SAR	Systemic acquired resistance
SDW	Sterile distilled water
SEM	Standard error of the mean
SUS	Sensitive/susceptible
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system

T4SS	Type IV secretion system
T6SS	Type VI secretion system
Tet	Tetracycline
Tc	Toxin complexes
TE	Tris-EDTA
Tm	Melting temperature
Vmax	Maximum growth rate
w/v	Weight/volume

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1 Introduction

1.1 Food security

With an ever-increasing global population and limited availability of natural resources, our planet is under increasing pressure to meet human and animal food requirements. Despite significant growth in food production over the past 50 years, nearly a billion people in the world still suffer from hunger and a greater number are malnourished. While the current world human population is 7.2 billion, the US Census Bureau projects a global population of over 9.2 billion by 2040, an increase of over 40 % (United Nations, 2015).

Currently, the world population is growing at an annual rate of 1.2 %, i.e. 77 million people per year. Six countries account for half of this annual increment viz. Bangladesh, China, India, Indonesia, Nigeria and Pakistan (Cohen, 2003). Because of the increasing world population, there will be a constant increase in demand for food across the globe that will persist for at least the next 50 years. It is estimated that farmers will need to increase yield by 1.5 % every year, representing a 35 % increase by 2030 and greater than 70 % by 2050. Meanwhile, several challenges like, climate change, limited natural resources and biodiversity restrict us from achieving increased intensification at the farm scale (Godfray *et al.,* 2010) . To compound the problem, most of the world's fertile land is currently in use and arable land areas cannot be expanded significantly. For reasons like this, the global challenge is to increase food production and quality with the resources available while minimising environmental impact. Improvement in pest control strategies represents one of the methods to generate higher quality and a greater quantity of agricultural products (Bale *et al.,* 2008).

1.2 Agriculture and Pest Control

To combat global food security issues, farmers must effectively control crop pests. These crop pests include insects, mites, nematodes, weeds, bacteria, fungi, viruses and vertebrates, which are responsible for:

- Up to 18 % loss of the world annual crop production (Oerke, 2005).

- Contributing to the loss of nearly 20 % of stored food grains (Bergvinson & Lara, 2004).
- Causing around US$100 billion damage to crops each year (Carlini & Grossi-de-Sa, 2002).

In the year 2015, out of the total Utilised Agricultural Area (UAA), 17.1 million hectares were used, which comprised of 70 % of land in the UK (Department for environment food and rural affairs, 2015) . Almost 36 % of UAA was considered to be croppable land, i.e. land currently under crops, bare fallow or temporary grassland. The total income from farming in the UK is estimated to be £3.8 million, making a significant contribution to the national economy. However, in 2011 30-40 % global crop production was damaged by pests (especially by weeds), pathogens and animal pests (Pimentel, 2009).

The most economically important animal crop pests are insects, predominantly because of their biological characteristics, abundance of species, high fecundity, and rapid reproduction. Phytophagous (plant eating insects) and mite pests are the main cause of agricultural losses, which can result in a 15.6 % decline in production (Leake, 2000). One of the main means of control of insect pests is through the use of synthetic insecticides. This control era began from 1930 with the introduction of DDT, BHC, Aldrin, Dieldrin, Endrin, and 2, 4-D. The use of organochlorines, especially DDT, was favoured for its broad-spectrum activity against many insect pests of agriculture. In addition to its ability to boost crop yields, DDT was cheap to manufacture, which led to rapid adoption of this insecticide across the globe, without complete knowledge of its long term environmental impact (Carvalho, 2006).

The extensive use of agrochemical pesticides was challenged by Rachel Carson in 1964, when she pointed out the risks of pesticides and showcased a picture of the environmental consequences of their careless use (Carson *et al.*, 1962). Carvalho (2006) showed that there are two opposite trends of the use of agrochemicals in the world. On one hand, the developed countries prefer to use fewer chemicals and more ‘‘green products”, to reduce the impact of pesticides on the environment. On the other hand, the use of cheap broad spectrum insecticides is still widesspread in developing countries, either because their patents have expired or because they are easy to synthesize.

To minimize the negative effects of these agrochemicals on the environment, it is crucial that we reduce the use of generic agrochemicals with less favourable environmental profiles and use pest-specific products and efficient cropping methods that reduce dependence on pesticides. Another issue that requires careful management is that due to the extensive use of a limited number of agrochemicals, insect pests have been subjected to a high degree of selection pressure (Brogdon & McAllister, 2004), which has resulted in the development of resistant pests.

As a result, an improvement in pest control is an essential component in addressing global food security issues while minimising environmental and human health issues.

1.3 Phytophagous Insects

Phytophagous insects are highly diverse comprising at least 500,000 species, which represents 40 % of all known insect species (Schoonhoven *et al.,* 2005). The herbivorous insects are divided into:

- Polyphages, which feed on plants of different families;
- Oligophages, which feed on plants of different species from the same family; and
- Monophages, which feed mainly on plants of one particular species.

There are phytophagous insect species in the majority of insect orders, including Orthoptera, Lepidoptera, Coleoptera, Heteroptera, Hymenoptera, and Diptera.

Many species from the above orders, including aphids, beetles, moths, mites, butterflies and other soil insects are significant pest of agriculture and horticulture and are estimated to be responsible for destroying one fifth of the world's total crop production annually (Sallam, 1999).

1.4 Aphids

Aphids are phytophagous insects characterized by the presence of piercing-sucking mouthparts, by which they acquire sap from the phloem of the plant (van Emden *et al.,* 1969). They belong to the superfamily Aphidoidea, in the homopterous division of the order Hemiptera. Aphids are considered as a major problem to agriculture, regardless of being a relatively small insect group of

4,000 species, compared to 10,000 species of grasshoppers, 12,000 species of geometrid moths and 60,000 species of weevils. Aphid species live mostly in temperate regions of the world where they colonize 25 % of the existing plant species (Schoonhoven *et al.*, 2005).

They may damage crops in several ways:

- Directly, through feeding on the phloem.
- Indirectly, through the production of honey dew which covers the leaves and may lead to faster aging of the leaf. Also, sooty moulds may develop on the honey dew, leading to reduced photosynthesis (Dedryver & Ralec, 2010).
- Indirectly, through the transmission of viruses.

1.4.1 Aphid morphology and life cycle

Aphids are small, pear-shaped insects with long legs and antennae. Most species have a pair of tube-like structures called cornicles projecting backwards out of the hind end of their body. The presence of cornicles distinguishes aphids from all other insects. Adults range in size from 1.5 to 2.5 mm long, depending on the species.

Aphids reproduce by cyclical parthenogenesis wherein clonal and sexual reproduction alternate within the annual life cycle. The actual reproduction rate depends on photoperiodic signals to generate a conformational change in order to facilitate each method of reproduction. Their life cycle begins with the hatching of eggs on a host plant at the beginning of spring (Figure 1.1). All the eggs that hatch produce aphid larvae and develop into founder females called fundatrices. These mature females reproduce asexually, often on secondary herbaceous hosts, during summer time. The aphids may start to reproduce sexually when autumn approaches and temperature falls. They are capable of sensing the differing light levels using sensory cells located on their cephalic region Once the change is detected signals are sent to the aphids ovaries, leading either to the production of haploid gametes, which require fertilisation, i.e. sexual reproduction, or in the absence of male gametes diploid oocytes initiate embryogenesis (Tjallingii, 2006; Le Trionnaire *et al.*, 2008).

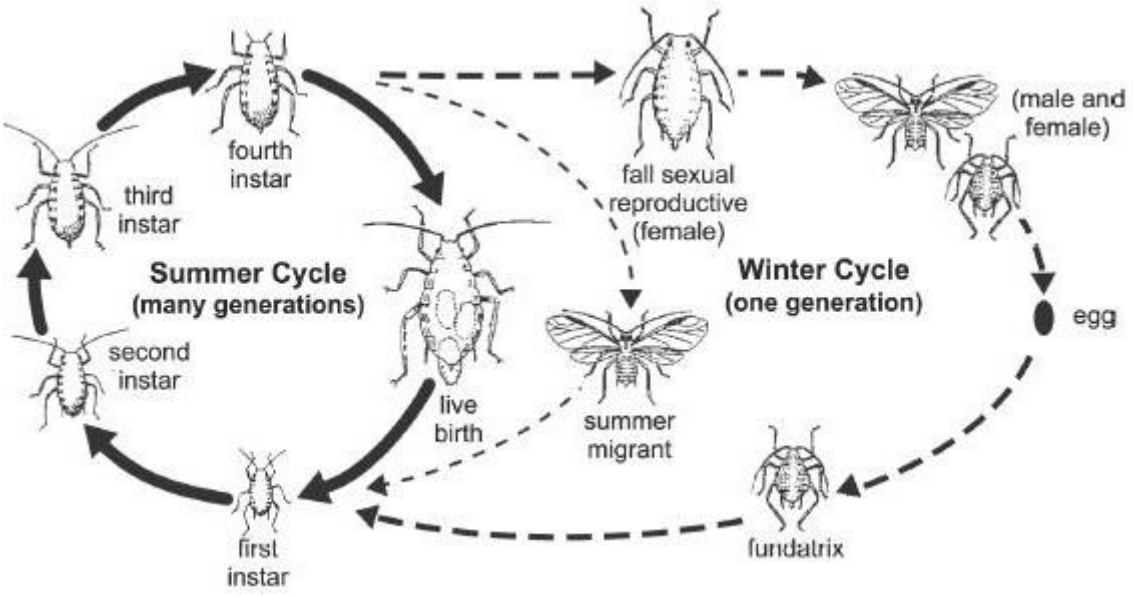

Figure 1.1: General life cycle of aphids. Asexual reproduction occurs during most of the year (summer cycle). Some aphid species produce a generation of sexual individuals that produce overwintering eggs as shown in the winter cycle.[Online image] Available http://www.420genetics.com/forum/showthread.php?t=703

In aphids, the two morphs, wingless and winged forms (Figure 1.2), occur in relation to environmental conditions. The phenotypic transition from wingless offspring to winged dimorph is determined mainly by the host plant quality (species and cultivar differences, nitrogen fertilization). The increase in aphid populations on the host plant results in crowding and a decrease in food quality and quantity, resulting in the production of winged forms (Mittler & Kunkel, 1971).

Figure 1.2: Aphid morph types. Mixed stages including winged and wingless adults of green-peach aphid (*Myzus persicae*) on *Prunus* leaf. [Online image] Available http://www.invasive.org/browse/detail.cfm?imgnum=2200053

The second factor involved in alteration to winged form is the introduction of natural predators to the host plant. Aphids are predated by a range of other insects belonging to the Coccinellidae, Neuroptera, Anthocoridae and Carabidae orders (Frazer, 1988). These predators along with

abiotic factors such as extreme temperature and rainfall conditions are responsible for decreases in aphid populations in summer (Brosius *et al.,* 2007). There are many experiments, which have demonstrated that the intentional introduction of natural predators into established aphid population can induce an increase in the proportion of winged dimorphs including when exposed to hoverfly larvae, lacewing larvae, adult and larval ladybirds and aphid parasitoid (Sloggett & Weisser, 2002). In order to address these mechanisms, Ladybirds were introduced to known aphid populations and despite there being an initial dramatic decrease in population, due to predation by the ladybirds, winged dimorphs could be observed after several days (Kunert *et al.*, 2005). Moreover, chemical signals, such as aphid alarm pheromone or a general enemy odour or enemy tracks, might lead to wing induction (Sloggett & Weisser, 2002). This indicates that aphids are able to sense a threat to their colony and adapt accordingly so that winged morphs are produced. This allows for the dispersal of the offspring to an area where the predator is not present. The ability of aphids to alter the phenotype of their offspring in response to the introduction of a predator facilitates the dispersal of a colony to other non-infested plants causing more widespread damage due to increased transmission of viruses to non-infected plants and crop damage (Kunert & Weisser, 2003).

1.4.2 Aphid Physiology

Aphids are plant sucking bugs. In contrast to chewing herbivores, which macerate plant tissue, they are adapted to feed on phloem sap. They are able to keep the cells they are feeding on alive while feeding, by preventing coagulation and keeping the sieve plate pores open. This allows aphids to feed from the same sieve element for several hours, even days (Tjallingii, 2006). An aphids stylus is comprised of 2 outer mandibles and 2 inner maxillae forming a salivary and food canal used to access the plants inner chemistry (Powell *et al.*, 2006). During feeding on phloem sap, continual gelling saliva is secreted, which forms a flange at the leaf surface (to limit stylet slippage) and a sheath that insulates the stylets from apoplastic defences. This gelling saliva also acts as a lubricating and hardening sheath for effective feeding (Walling, 2008). To ensure competent feeding in host plants, aphids should counteract the plant's defence response and

participate in the manipulation of plant responses to ensure compatible interactions between aphids and host plants (Giordanengo *et al.*, 2010). The phloem sap is toxin free with high concentrations of sugars, which provides an abundant source of carbon, energy and nitrogen (predominantly in the form of free amino acids) (Douglas, 2006). Certainly, phloem sap provides a rich diet to aphids, but the aphids have to overcome the 'nitrogen barrier' and 'sugar barrier' for sap utilization. The amount and composition of nitrogen is crucial for optimal growth and fecundity in aphids. Moreover, this problem is significant in aphids because they are metabolically impoverished, lacking the ability to synthesize 9 of the 20 amino acids that constitute whole protein. The essential amino acid content of phloem sap is insufficient to support the observed growth rate of the aphids (Karley *et al.*, 2002) . Aphids overcome this nitrogen barrier by living in symbiosis with the bacterial species *Buchnera*. *Buchnera* resides in the gut of the aphids and are able to synthesise the amino acids that the aphids are not able to extract from the plant (Douglas, 2006). Aphids overcome the 'sugar barrier' by an osmoregulation process where there is a continuous flow of fluid into the gut at high osmotic pressure and as a result aphids shrivel while they are feeding (Douglas, 2006).

1.4.3 Aphid Immune system

Aphids have to survive with a wide array of pathogens in their environment. These include parasitoid wasps which consume their hosts as they develop inside their body, and a variety of viral, bacterial and fungal pathogens. As compared to vertebrates, which utilize acquired immunity and the more classical innate immunity, aphids exclusively depend on innate immune mechanisms for their defence (Lemaitre & Hoffmann, 2007). These innate mechanisms consist of cellular and humoral components, which comprises entrapment of invading pathogens in clots, phagocytosis by immune-competent cells (haemocytes) and death, via induced production of antimicrobial peptides and Reactive Oxygen Species (ROS) (generated, for example, by nitric oxide synthase and phenol oxidase) (Lemaitre & Hoffmann, 2007).

In the model insect system *Drosophila melanogaster*, recognition of an invasive microbe leads to signal production via four pathways: the Toll, Immune-Deficiency (IMD), c-Jun N-terminal Kinase

(JNK), JAK/STAT and JNK signalling pathways (Lemaitre & Hoffmann, 2007). Such signalling triggers the production of a multitude of effectors, including Reactive Oxygen Species (ROS) and antimicrobial peptide production. However, additional genes, such as the fork head transcription factor FOXO (Becker *et al.*, 2010) and recognition factors of cell and tissue have been identified regulating insect innate immune responses.

The pea aphid, *Acyrthosiphon pisum*, genome represents a valuable model system to study molecular interactions of a host with both beneficial and harmful microbes (Richards *et al.*, 2010) due to two reasons:

1. To date information regarding insect immune and stress responses comes only from holometabolous insects such as files, beetles and butterflies. Therefore, the hemimetabolous pea aphid provides insight into immunity and defence in more basal, non-holometabolous insects, which have incomplete metamorphosis.
2. Recent genome sequencing of the *A. pisum* and their well-studied associations with both obligate and facultative bacterial symbionts for survival (Moran *et al.*, 1998; Sandström *et al*., 2001; Moran, *et al*., 2005).

Interestingly, recent studies have provided evidence that the pea aphid, *A. pisum*, has a reduced immune repertoire when compared with Drosophila and other investigated insects (Richards *et al.,* 2010).

The pea aphid genome contains a number of genes that underlie immune responses in other insects (e.g. Toll and JAK/STAT pathway genes). In contrast, several genes that are thought to be critical for immune function against bacterial pathogens are missing. *A. pisum* lacks typical insect antibacterial peptides (including defensins, attacins, and cecropins) and essential genes involved in the IMD pathway (including peptidoglycan recognition proteins (PGRPs) and the central IMD protein itself (International & Genomics, 2010). Both transcriptomic and proteomic analysis revealed few up regulated genes/proteins in microbe exposed pea aphids compared with unchallenged aphids (Altincicek *et al.,* 2008). Furthermore, immunological analysis has

demonstrated that *A. pisum* displays only weak lysozyme-like activity, hemolymph coagulation, and phenol oxidase activation reactions (Laughton *et al.*, 2011).

While these studies postulated that pea aphids have a reduced immune system as compared to other characterized insects.

1.4.4 Aphids and damage to crops

Aphids are responsible for considerable crop losses both directly and indirectly (see above). Aphid feeding on flowers of fruit trees (e.g., *Myzus persicae* on peach trees) can lead to bad fructification (flower abortion). They also cause the malformation of fruits on crops such as peppers and strawberries and leaf damage on crops such as lettuce and cabbage. At higher aphid population, such kind of damage frequently increased by honeydew excretion, which covers the leaf surfaces and turns black by the growth of sooty mould fungus (Figure 1.3). The formation of sooty moulds hinders photosynthesis of plant and affecting the marketability of the produce. On cotton crops, the main cause of the ‘‘sticky fibre’’ symptom is the penetration of honeydew produced by *Aphis gossypii* in the open mature boll. In addition many aphid species are highly efficient vectors for a large number of plant viruses that in many instances can cause greater damage to their host than via direct feeding (Kennedy *et al.*, 1962).

Figure 1.3: Leaf curling in a Peach tree caused by *Myzus persicae* . [Online image] Available http://www.invasive.org/browse/detail.cfm?imgnum=1326232

1.4.5 Virus transmission

The structure of aphid mouthparts, their searching behaviour for host plants, the range of available host plants and high reproductive rates contribute to the efficiency of aphids to act as virus carriers. Aphids are vectors of several viruses that severely affect several different commercial food crops.

The Green Peach aphid (*Myzus persicae*) has been found to be the most effective aphid vector in transmitting viruses; however widespread transmission of the Cucumovirus is possible due to the fact that 26 different aphid species are able to carry and transmit this virus (Chen & Francki, 1990). It is estimated that over 100 different viruses can be transmitted by *M. persicae*, many on important crops such as beans, sugar beet, sugarcane, brassicaceae, potato and tobacco (Kennedy *et al.*, 1962). Among the most damaging plant viruses dispersed by the green-peach aphid, there are two important pathogens of the Solanaceae family: Potato Leaf roll Virus (Eskandari *et al.*, 1979; Van den Heuvel *et al.,* 1991) and Potato Virus Y (Hoof, 1980). *M. persicae* is also responsible for the transmission of Beet Western Yellows (Brault *et al.*, 1995) and Beet Yellows Viruses (Sylvester, 1956) to Chenopodiaceae; Lettuce Mosaic Virus to Compositae (Dinant & Lot, 1992); Cauliflower Mosaic Virus to Cruciferae (Day & Venables, 1961) and Cucumber Mosaic Virus to Cucurbitaceae (Gallitelli, 2000).

Other important viruses spread by aphids are Circoviridae, Luteoviridae, Rhabdoviridae and Umbraviruses (Van den Heuvel *et al.*, 1994). A Luteoviridae, known as potato leaf roll virus, causes significant damage in many host species, causing leaves to roll and turn a pale yellow colour. Later during the infection, leaves become stiff, dry, leathery and crispy. The horticultural crop, pepper, suffers great loss worldwide, mainly due to viral infection (Gorsane *et al.,* 1999) particularly Polyviruses, a group of viruses that include Pepper mild mosaic virus, Pepper venial mottle virus and the Peru tomato virus (Green & Kim, 1991). The damage caused by these three viruses depends on the strain and severity of the infection; they are a particularly problematic group of viruses as the pesticides currently available in the market are unable to successfully control the spread of these diseases.

The two broad mechanisms of aphid transmitted plant viruses are; non-persistent and persistent or circulative (Hogenhout *et al.*,2008). In non-persistent transmission, aphids can inoculate the virus into plants for only a few minutes after acquisition. The insect loses the virus within a few minutes and upon moulting in the case of Polyviruses (Ammar, 1994). In persistent transmission, aphids can inoculate the acquired virus for much longer periods (days or weeks), transmitting the virus after moulting and often for their entire lifespan (larvae or/nymphs into adults) as reported for Luteoviridae, Geminiviridae and Nanoviridae families (Hogenhout *et al.,* 2008).

Finally an intermediate category of semi-persistent viruses exist; these can be transmitted by the vector from a few hours to a few days post-acquisition, but are lost after moulting. Non-persistent viruses are retained by the vector mainly in the stylet (food canal), whereas semi-persistent viruses are retained mainly in the foregut (Ng & Falk, 2006).

1.5 Aphid control strategies

In order to control aphid infestation, considerable effort has gone into developing effective, host specific pesticides that cause minimal damage and disruption to the environment and ecosystem. Control of aphids relies heavily on the use of synthetic insecticides augmented by a few other strategies, including biological control (especially in contained environments). The prevalent agrochemicals used in the control of aphids include carbamates, organophosphates, pyrethroids, neonicotinoids, and antifeedants such as flonicamid/pymetrozine (Bahlai *et al.,* 2010). Most of the major classes of insecticides for aphid control act on different targets of the central nervous system, leading to the disruption of nerve impulse transmission and death. The first insecticide efficient against aphids was 'natural' nicotine, extracted from tobacco leaves during the Second World War that kills aphids by direct contact. After the war, chemical control of aphids developed rapidly with the introduction of organ chlorinated compounds in the late 1940s, organophosphorus in the 1950s, carbamates in the 1960s and pyrethroids during the 1970s. During this period a number of selective pesticides were registered, such as certain organophosphates (e.g., phosalone) and carbamates (pirimicarb) and aldicarb (Temik). Foliar

spray of these insecticides provides only marginal control of aphids due to their short residual activity under field conditions. However, foliar spray of pymetrozine (neuroactive insecticide) provided excellent aphid control by modulating insect chlordotonal organs. At the end of 1980s, a new systemic class of insecticides, the neonicotinoids (imidacloprid (Admire) and thiamethoxam (Platinum), were introduced for seed treatment control. These insecticides are spread inside the infected plants through the xylem and phloem and are able to prevent certain virus transmission while causing little harm to natural predators.

However, the long-term use of any insecticide is continually threatened by the ability of insects to evolve resistance that renders the chemicals ineffective. Such resistance poses a serious threat to insect pest control both in the UK and throughout the world.

1.6 Insecticide Resistance in Aphids

M. persicae has developed resistance to numerous insecticides through either metabolic or target site mechanisms:

1) **Metabolic**: Increased production of detoxifying enzymes (such as esterases and P450s) that metabolise or sequester the insecticide before it reaches its target protein. This form of resistance has been demonstrated for organophosphates and neonicotinoids, although carbamates and pyrethroids are also affected to a lesser extent.
2) **Target site resistance mechanisms**: Is caused by structural changes (mutations) in the insecticide target site that results in a decreased affinity for the insecticide. The three target site mutations that have been reported in *M. persicae* are:
    - **MACE (Modified Acetylcholinesterase)**: A mutation in the organophosphate and carbamate binding site acetylcholinesterase with the resistant modified enzyme (MACE) conferring high resistance specifically to carbamates, pirimicarb and triazamate (the latter is now not used in the UK).
    - **Knockdown resistance or Kdr**: This type of resistance is conferred by one or more point mutations in the target site of pyrethroids, the voltage-gated sodium channel in the nerve

axon membrane. Kdr resistance is associated specifically with resistance to pyrethroids and DDT.

- **Neonicotinoid resistance/R81T**: This form of resistance is conferred by a mutation at a key position in the neonicotinoid target, the nicotinic acetylcholine receptor, and confers high levels of resistance to all neonicotinoids

Worryingly, it is now commonplace to find populations (and indeed individuals) of *M. persicae* carrying many of the resistance mechanisms detailed above resulting in multiple resistance to a range of insecticides and severely limiting control options. One potential route to restore susceptibility in these populations in the absence of strong selection would be fitness costs associated with these mechanisms, for example,

occurrence of significant fitness penalties associated with high esterase resistance mainly during stress, such as lower survival during cold and wet weather, lower fecundity and reduced motility from senescing leaves (Foster, Kift, *et al.,* 2003).These fitness cost penalties effectively control the frequency of insecticide-resistant forms in countries such as UK. Nevertheless, they become less significant in protected or semi-protected cropping systems where there are no reports of aphid damage. As a consequence, these conditions are likely to support insecticide-resistant *M. persicae,* resulting in the development of effective biocontrol against these forms.

If resistance completely compromises the use of chemical pesticides there may be alternative ways to control aphid pests. These include, the use of light oil sprays including mineral, neem and garlic oil, which revealed initial promising results (Perring *et al.,* 1999). However, for effective aphid removal several applications of the sprays were needed throughout the plants life. With the knowledge that the aphids are attracted to colours between the wavelengths 500-700nm, experiments have also been performed using coloured sticky traps. A yellow glass filled with few drops of soap and water is placed to attract aphids and allowed them to drown. Additionally, sticky substance, such as double-sided tape around yellow sheet of paper or board is also used for trapping aphids.Trials using these showed that the traps reduced the spread of aphid transmitted diseases, but were not successful in completely eradicating the spread of the viruses (Heinz *et al.,*

1992). The use of beneficial organisms, known as 'beneficials' and natural predators on aphids have proven to be the most successful alternatives compared with the use of chemicals.

1.7 Biological control

The continuous threat of insecticide resistance in many insect pests and current restrictive legislation associated with chemical pesticides, means alternative means of control are now urgently required.

Biological control practices can be divided into two broad categories:

1.7.1 Classical bio-control

In classical bio-control, an exotic control agent is intentionally introduced into a new geographic area with the goal of long-term establishment. One of the most successful examples of classical biological control was with the cottony cushion scale, a pest that was devastating the California citrus industry in the late 1800s. A predatory insect, the vedalia beetle and a parasitoid fly were introduced from Australia (Hajek *et al.,* 2007). Classical bio-control is referred to as long term control because the introduced insect species have escaped from natural predators that normally regulate populations in its area of endemism (= the 'enemy release hypothesis') and it is anticipated that the invasive pest will be naturally controlled once reunited with its natural enemies (Keane & Crawley, 2002).

Regarding biological control, several studies have proved that there are some natural enemies that could be used to reduce aphid population density. Many aphid antagonists have been described in the past, such as ladybird beetles (order Coleoptera), green lacewings (Neuroptera), wasps (Hymenoptera) and hoverflies (Diptera) (Brewer *et al.,* 2005) Most of them are general predators of multiple aphid species or even other insect species and are often conditioned by the environment and the host plant (Tamaki *et al*., 1981). The pupae of *Aphidoletes aphidimyza* (order Diptera, family Cecidomyiidae), also known as the aphid midge; has been commercialized as a pest control solution for greenhouse crops, as it was demonstrated that this insect is able to reduce growth of green-peach aphid populations and their resulting damage on peppers

(Gilkeson, 1987) and tomatoes (Meadow *et al.,* 1985), both in greenhouse conditions. Furthermore, this natural enemy was also able to control green-peach aphid populations on field-grown peppers (Meadow *et al.*, 1985).

Other commonly used 'beneficials' include syrphid (hoverfly), Cecidomyiidae (gall midge), chrysopid larvae (lace wing larvae), coccinellids (ladybirds), carabids, spiders, hymenopteran parasitoids (parasitic wasps) and entomophagous fungi (Kunert & Weisser, 2003; Schmidt *et al.,* 2003). These are introduced with the intent of permanently establishing a population of natural enemies to reach an equilibrium at which the aphids are kept at a level that does not cause excessive damage to the crop but also provides enough food to keep the beneficials at an appropriate level.

1.7.2 Augmentation bio-control

Augmentation biological control is more efficient way to manage release of natural enemies. Two general approaches of augmentation have been employed: mass production and periodic colonization; or genetic enhancement of natural enemies. The foremost approach is most common, which involves large scale production of natural enemies in insectaries and then released either by inoculation or inundation. Inundation involves release of large numbers of natural enemies that results in high mortality of the pest population. An inoculative release is another way of augmentation which involves periodic releases of natural enemies before occurrence of pest populations in each growing season. Since, seasonal release of natural enemies in each spring helps them to build up population and control the pest population in growing season. Examples includes periodic releases of the parasitoid, Encarsia formosa to control whiteflies, leafminers, thrips, aphids and mites in greenhouses and parasitoids such as *Trichogramma* are regularly released in large numbers (Inundative release) (Lenteren & Bueno, 2003; Lenteren, 2000).

The critical evaluation of augmentation described a 64 % failure rate in many control cases and a large amount of cost is associated with managing this kind of control method, which is higher than insecticide use (Collier & Steenwyk, 2004). The main reasons for such a low success rate are

unfavourable environmental conditions, compensatory mortality, enemy dispersal, host refuges from released natural enemies, and predation of released agents (Collier & Steenwyk, 2004).

Successful augmentation generally requires advanced planning, biological expertise, careful monitoring, and optimal timing of release, patience and situations where certain levels of pests and damage can be tolerated. This can be expensive if the area that needs protecting is large as more than one application of the beneficial will nearly always is necessary. In addition to this, invasion of predators also stimulates alarm pheromone in aphids which results in winged morphs. As a result, the winged morphs reproduce and allow dispersal of aphid populations to larger areas. Therefore, in order to achieve effective pest control for such polyphagous pests, alternative, cost effective pest control methods are required.

1.8 Microbial pesticides

Microbial control is a form of biological control that uses pathogenic microbes such as yeast, bacterium or virus, or toxic microbial products such as proteins. This is a less recognised practice but is becoming more prevalent as more research and development work is carried out (Kaya & Lacey, 2007).

Microbial insect pathogens may be divided into two groups according to mode of entrance and action in the host:

1. **Through Ingestion**

Ingestion of bacteria, viruses and protozoa with food that cause infection and ultimately death are known as stomach poisons. In general, bacteria cause damage by replicating inside host tissues and secrete toxins or other virulence factor during colonisation. Viruses multiply in specific tissues of host and exploit host cell machinery for their own benefit (Table 1.1).

2. **Through the Integument**

Many pathogenic fungi enter their host tissues through the insect cuticle. Once fungus penetrates through insect cuticle, it starts invading the host circulatory system (hemolymph) by formation of

germ tubes and penetration pegs. Such spread of infection and utilization of host nutrients caused death (Table 1.1).

Table 1.1: Insect pathogens used in bio-control.

Details	Pathogen	Host
Bacteria	*a) Bacillus thuringiensis*	Lepidopterous pest
	b) Photorabdus luminescens	Most insect larvae
Viruses	a) Baculoviruses (br)	Lepidopterous pest
	b) Granulosis viruses (Gr.)	Mosquitoes, mites etc.
	c) Nuclear Polyhedrosis Virus (NPV)	Lepidopterous pest
Fungi	a) *Lagenidium giganteum* b) *Verticillium lecanii* *c) Beauveria bassiana*	Lepidopterous pest, beetles, aphids, scales, mites etc.
Protozoa	*a) Nosema locustae*	Grasshoppers, Orthoptera
Nemtaodes	a) *Steinernema spp*.	White grub

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Microbial insect control strategies (Nicholson, 2007):

- Should be environmentally safe
- Should have broad spectrum specificity against insect pests but not against beneficial insects
- Should be nontoxic to human health and
- Should be cost effective

Considering the above factors, beneficial microorganisms from the disease suppressive soils and 'no disease' plant surfaces have been particularly interesting avenues for research.

1.8.1 Plant beneficial microorganisms

The plant-beneficial microorganisms, mostly present in disease-suppressive soils compete effectively with pathogens for rhizosphere niches and nutrients. These disease suppressive strains produce a number of secondary metabolites including phenazines, 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, cycliclipopeptides, siderophores and hydrogen cyanide, which offer selective and competitive advantages. PGRPs reduce the severity of many fungal diseases and soil borne pathogens directly by antibiosis or indirectly by the induction of plant defence mechanisms (induced systemic resistance, ISR) (Haas & Keel, 2003; Raaijmakers *et al.,* 2010; Jousset *et al.,* 2011). Many root colonising pseudomonads act as natural suppressive agents to specific soil borne diseases such as take-all of wheat, black root of tobacco, and rhizoctonia root

rot of sugar beet (Haas & Defago, 2005; Mendes *et al.*, 2011; Weller *et al.,* 2012; Almario, *et al.,* 2013).

1.8.2 Entomopathogenic microorganisms

In addition to disease-suppressing microorganisms, insect-killing bacteria have been found in agricultural soils (Bode, 2009). Insects are a highly diverse group in the animal kingdom, and certain parasites are specifically adapted to insects as a host and/or food source (Bode, 2009). The well-known bacterial pesticide *Bacillus thuringiensis* (Bt) is a Gram-positive bacterium native to soil and its products have been used commercially by growers as an insecticide for over 50 years. Besides *B. thuringiensis,* other bacteria such as *Pseudomonas entomophila, Photorhabdus* spp. and *Xenorhabdus* spp., carry genes encoding insecticidal secondary metabolites (Duchaud *et al.,* 2003; Vodovar *et al.,* 2006; Olcott *et al.,* 2010)**.**

1.8.2.1 *Bacillus thuringiensis*

Although Bt (*Bacillus thuringiensis* formulated as a biopesticides) is considered as an important biopesticide for controlling pests, it accounts for around 2 % of the total market of insecticides (Bravo *et al.,* 2011)

There are over 500 *cry* genes for the production of these proteins and based on their primary amino acid sequence they are separated into 4 structurally different families: 3 domain Cry toxins (3D), mosquitocidal Cry toxins (Mtx), binary-like toxins (Bin) and the Cyt toxins (Bravo *et al.,* 2005). Both Cry and Cyt toxins are very selective and cause toxicity in the members of the lepidopteran, coleopteran and dipteran family.

In the earlier studies Bt toxins showed a low level of toxicity against aphids due to the use of toxin crystals or spore suspension in feeding assays rather than pre-solubilized toxins (Payne & Cannon, 1993; Walters & English, 1995). Later, solubilized forms of four Cry δ-endotoxins (Cry1Ab, Cry3A, Cry4Aa and Cry11Aa) displayed a negative impact on the survival of pea aphid and retarded the growth of survivors (Porcar *et al.,* 2009). However, these toxins showed greater aphid toxicity than previously reported, although the toxicity levels were still low compared to the toxicity of some of the Cry toxins used for lepidopteran and coleopteran pest management in the field.

Vegetative insecticidal protein (Vip) purified from Bt isolates, showed insecticidal activity against the cotton aphid, *Aphis gossypii*. This binary toxin, Vip2Ae-Vip1Ae, bound to a 50 kDa receptor from cotton aphid brush border membrane vesicles (BBMV), but did not bind to other lepidopteran gut receptors indicating that the toxin may have aphid specificity (Sattar & Maiti, 2011). Another study characterized novel cry related protein {40 % pairwise identity to the cancer cell killing Cry proteins parasporins Cry41Ab1 and Cry41Aa1} which exhibited toxicity specifically to green peach aphid, *M. persicae* (Palma *et al.,* 2014). Gene sequencing, annotation and the adjoining analysis led to the discoveries of novel cry genes encoding for large proteins to which insecticidal activities are attributed. The results indicated putative novel insecticidal protein gene 1143 bp long was found in two *B. thuringiensis* strains (Leapi01 and Hu4-2), whose sequences exhibited 100 % nucleotide identity (Palma *et al.,* 2014).

It is not only possible for the toxic products of this bacterium to be used as a defence against insect pests but plants have also been engineered to express the cry genes (Wu *et al.*, 1997). Bt crops, including potato, cotton and corn are all commercially available (Bravo *et al.,* 2011).

1.8.2.2 *Photorhabdus spp.* and *Xenorhabdus spp.*

Entomopathogenic bacteria such as the Gram-negative bacteria *Photorhabdus luminescens (Ph. luminescens)* and *Xenorhabdus nematophilus* that live as mutualists in the intestines of entomophagous nematodes have developed different strategies to interact with and kill insects. The mutualistic bacteria released in the haemocoel, start multiplication with toxin production and kill the insect host within 48 h (Bowen *et al.,* 1998). Genome analysis revealed several genes encoding for large proteins to which antibiosis and insecticidal activities have been recognized (Waterfield, *et al.,* 2001; ffrench-Constant & Waterfield, 2006; ffrench-Constant *et al.,* 2007). Three different classes of insect toxins were reported in the *Photorhabdus luminescens* genome. The large orally active toxin complexes (Tc) are displayed on the outer membrane of the bacterium and require all three components (ABC) for full toxicity (Bowen *et al.,* 1998; ffrench-Constant *et al.,* 2007). Another type of toxin exhibits pro-apoptotic activity and was named as, 'makes caterpillar floppy' (Mcf) toxin. The injectable activity of this toxin results into the insect

losing its body turgor entirely and becoming 'floppy' (Daborn *et al.,* 2002; Waterfield *et al.,* 2003). A third class of toxin described as '*Photorhabdus* insect-related' (PirAB) binary toxins have showed oral and injectable toxicity in some insects (ffrench-Constant *et al.,* 2007).

1.8.2.3 Other Entomopathogenic bacteria

One recent investigation indicated that *Pantoea stewartii*, an enteric phytopathogen, can kill aphids and the candidate gene, you cannot pass (YCP1), is responsible for pathogenicity in the aphids. On further analysis, this study discovered that YCP1 belong to RHS/YD repeat family of proteins which involved in bacterial adhesion and aggregation. After ingesting the bacteria, solid aggregates formed in the aphid gut. This led to reduced honey dew production, a cessation in feeding and thus starvation in the infected aphids (Stavrinides *et al.,* 2010).

The plant pathogenic bacterium, *Dickeya dadantii* is also categorized as an aphid killing bacteria. The mode of infection is ingestion and the bacteria multiply in the aphid gut. As early as one day post-infection, they invade the gut epithelium and circulate in the haemocoel (body cavity) of the insect, with a specifically localize in the fat body. As infection continues to spread the other organs, such as the brain or the embryos, and death is provoked by septicemia (Grenier *et al.,* 2006).

1.8.2.4 Entomopathogenic pseudomonads

Insect associated soil-inhabiting bacterium *Pseudomonas entomophila (P. entomophila)* has exhibited oral toxicity to *Drosophila* and some lepidopteran insects without the need of a vector such as a nematode. The presence of insecticidal toxins (*Photorhabdus* Tc components) and secretion of metalloprotease AprA, to degrade antimicrobial peptides in the insect gut supported their role in insect pathogenicity (Vodovar *et al.*, 2006).

Vodovar *et al.* (2006) sequenced the complete genome of *P. entomophila* and reported several putative genes for insecticidal proteins and no genes encoding for the type III or type IV secretion system were found.

Pseudomonas syringae pv. *syringae* (*Psy*) B728a is also considered pathogenic to aphids. The presence of *tc*-like genes in the *P. syringae* assumed that this bacterium interacts with an

unknown insect vector. Although, the presence of the *tc*-like genes might indicate it interacts with a wide range of hosts other than insects (ffrench-Constant *et al.,* 2007). The mutagenesis screen led to the discovery that the *Psy* B728a *fliL* mutant, defective in a gene required for flagellum formation and motility, was also hypovirulent and caused a significant decrease in the numbers of aphids affected by the bacteria. The results indicated that both *fliL* and the associated swarming phenotype have an important role in regulation of virulence specific genes that contributes to aphid colonization and death (Stavrinides *et al.,* 2009).

The well-characterized root-colonizing disease-suppressive agents, *P. fluorescens* CHA0 and the related strain Pf-5 exhibit potent insecticidal activity. The insecticidal activity is associated to a genomic locus encoding a large protein toxin termed Fit (for *P. fluorescens* insecticidal toxin) that is related to the insect toxin Mcf of the entomopathogen *Phluminescens*, a mutualist of an insect-invading nematode (Péchy-Tarr *et al.,* 2008). The occurrence of insecticidal activity in the plant colonizing Pseudomonads is somewhat unexpected as these bacteria have no known insect association. As the soil environment is also rich in invertebrates, there is a possibility that invertebrates can feed on soil microbes either through ingestion of particulate soil matter or other organic material. If bacteria can survive ingestion then the bacteria may recycle back to the soil reservoir. Another classical route of dispersal of entomopathogenic bacteria is that they are carried out by wind in aerosols or rain splash or windblown rain which strike aerial surfaces of non-infested plants. After arrival on the plants, these versatile strains may function as insect pathogens and switch between insect hosts and the plant environment. It is hypothesized that Fit toxins may be part of collection of toxic exoproducts that help these Pseudomonads improve their ecological competitiveness and defence against predators (Péchy-Tarr *et al.,* 2008). The Fit insect toxin exhibited 73 % identity with the insecticidal toxin Mcf1 and 67 % with Mcf2, of *Ph luminescens*, a bacterial symbiont of entomopathogenic nematodes. Previous research characterized toxin complex gene clusters and insect toxicity among *P fluorescens* group (*P. chlororaphis, P. corrugata, P. koreensis, and P. fluorescens* subgroups) and indicated the capacity to kill insects by both FitD-dependent and independent mechanisms (Rangel *et al.,* 2016). The

results demonstrated *fit* cluster (or Fit cluster), which is highly conserved among the strains and Tc gene clusters are inherited through a complex process involving horizontal gene transfer as well as vertical transmission through defined lineages of *Pseudomonas.* All these investigations have demonstrated the role of plant-associated bacteria in killing insects and their potential utility in biocontrol.

One study has shown that the phylloplane (from leaf surfaces) may be a promising source of bio-control agents (Andrews, 1992). Although, few microbes have been isolated from plant tissues, many more can be recovered from their phylloplane, including bacteria, fungi and yeasts. Bacteria are the predominant microorganism found on the phylloplane, with cell density averaging between 10^6-10^7 per cm^2 (Hirano & Upper, 2000). Some of these bacteria have been shown to be pathogenic to insects, such as aphids, that feed on plants (Maji, 2004; Sowndhararajan *et al.,* 2013). Another study demonstrated native bacteria populations either isolated from the rhizosphere or from the phylloplane can reduce pathogen populations on the leaves. They suggested direct antagonism mechanisms, production of secondary metabolites by antagonists and ability to trigger induced systemic resistance response in plants were involved in reduction of bacterial blight severity on passionfruit plants (Halfeld-Vieira *et al.,* 2015).

The utilization of naturally occurring bacteria as pesticides may help achieve effective, environmentally safe pest control that can be applied directly on the crops. Currently used microbial bio pesticides don't show evidence of mammalian toxicity - infections are rare and the bacteria generally need to be introduced directly into the circulatory system. However, any bacteria that are being applied to a crop may have adverse effects on beneficial insects that may interact with the crop such as bees and other pollinators since their host specificity is still not fully characterised.

1.9 Integrated Pest Management

The Integrated Pest Management (IPM) concept was introduced to reduce the amount/ frequency of pesticides used in order to secure a more sustainable plant production. IPM is an ecosystem-based strategy that focuses on long term control of pests through different combinations of control measures, including improved cultural practices, mechanical and physical controls, biological control, habitat manipulation, chemical control and use of resistant varieties (Pedigo, 2002).The use of particular chemical pesticides within certain limits is allowed and minor losses are allowed to acceptable economic levels in order to minimise risks to human health and environment.

IPM management proposed five basic approaches to improve insect management:

1. Identification of pest – To evaluate insect types.

2. Monitor level of pest population and assessing damage

3. Follow standard procedure for management action – In general IPM, economic injury thresholds where proper action should be taken

4. Implementation of appropriate treatment through use of physical, cultural, biological, or pesticide controls, or management tools

5. Assess the effect of pest management after implementation.

The above stated strategies have been employed in different IPM management practices to suppress pest activities rather than eradicate the pests. IPM examples: In glasshouses, weeds like Queen Anne's lace (*Daucus carota*) or London rocket (*Sisymbrium irio*) are grown to accommodate beneficial insects such as lacewing or lady beetle larvae which maintain aphid populations at acceptable levels. Another illustration of IPM relates to growing tomatoes in a glasshouse where, to control whitefly, Encarsia wasp is introduced; for the red spider mite, Phytosseiulus; and for the leafminer, Diglyphlus. Additionally, the introduction of the meat eater, Macrolophus insect, is used to make sure that pest populations should be maintained at satisfactory levels.

In summary, the main aspect of IPM is to achieve sustainability in agriculture and prevention of major outbreaks of pest populations. Hence, future approaches should be combinations of prevention and therapy to minimise pest damage. In this direction, the designing of molecular markers to select resistant varieties, habitat manipulation, insecticide resistant predators/parasitoids, novel entomopathogens, organic farming, proper use of semiochemicals and the use of information technology would enable help in building up healthy crop environment.

1.10 Aims of the project

An aphid is a globally important pest causing direct damage to a broad range of arable and horticultural crops and transmitting more than 100 plant viruses. Because resistance has rendered many of the insecticides used for control ineffective there is an urgent need to develop alternative means of control.

The plant surfaces harbour a variety of microbes which enable the plant to deal with abiotic and biotic stress via growth promotion and induced systemic resistance. Some bacteria found on leaf surfaces possess pathogenic qualities and are known to kill aphids and other insects. Therefore, there is potential to either use them, or their products, as a directly applied biological control agent or to manipulate the crop environment to enhance their development.

In the Livermore (2016) study, 140 bacteria were isolated from the phylloplane and rhizosphere of a range of plants; nine of these bacteria were observed to be pathogenic to aphids. This raised the total of aphid killing bacteria to 14 when including five other bacteria from a Fabrizio study (Alberti, 2011).

The initial screening of aphid toxicity using all 14 bacteria showed them to be pathogenic to *M. persicae* (green peach aphid), Lettuce aphid (*Nasonovia ribisnigri*), glasshouse potato aphid (*Aulacorthum solani*), cabbage aphid (*Brevicoryne brassicae*), lupin aphid (*Macrosiphum albifrons*) and pea aphid (*Aphis fabae*) with different mortality rates. To evaluate the potential of bacterial pathogens to cross the species barrier and infect new hosts, further assays were performed to

explore host range. Host specificity tests of the aphid-pathogens were carried out on different insect species: *Oryzaephilus surinamensis*, *Sitophilus oryzae*, *Galleria mellonella*, *Cryptolestes capillulus* and several Lepidoptera species. The results showed no death was observed when the tested insects were exposed to the bacteria. This suggests that all the bacterial pathogens were restricted to killing aphid species.

Further characterization of the bacterial pathogens using biochemical and histochemical tests such as antibiotic resistance testing, suggested that the majority of bacterial strains isolated were resistant to both ampicillin and Nitrofurantoin. The identification of the bacterial strains was carried out by 16S rRNA gene sequencing. On comparative analysis of all bacterial strains, the three most promising strains named "*Pseudomonas poae* PpR24", *Pseudomonas fluorescens* PfR 37" and "*Citrobacter werkmanii* CwR94" were sent for whole genome sequencing. The screening for potential insecticidal genes in the sequenced genomes was done via a database search engine designed by Prof. Primitivo Caballero's lab (Universidad Publica de Navarra), which confirmed the presence of different insecticidal toxins such as Tc (Toxin complexes), Rhs (rearrangement hotspot) element and Fit toxin. Other investigations were carried out into methods of pathogenicity, and potential application methods, with the hope of identifying suitable bacteria for use as a pesticide (Livermore, 2016).

The current study builds on the above work that identified plant-residing bacteria, isolated from non-infested plants, which are pathogenic to diffferent species of aphid (Livermore, 2016). The main aim of this project is to characterise these potential aphid killing pathogens in much greater detail. If these pathogens are to be utilized as bio-control agents, then it is essential to identify the targets affected by the pathogen and investigate how the bacteria can kill aphids. The specific objectives of the current work were:

1. Identification of bacterial isolates which can kill different insecticide resistant aphid clones (Chapter-3).
2. Examine baseline bacteria susceptibility of different insecticide resistance aphid clones and observe any fitness costs associated with known resistance mechanisms. (Chapter-3).

3. To characterise plant-bacteria interactions in order to achieve reduced aphid populations on model crop plants. (Chapter-4).
4. Study aphid behaviour in response to bacteria treated plants. (Chapter-4).
5. To examine the fundamental interaction of bacteria and aphid using transcriptomic analysis to identify changes in gene expression associated with exposure to bacteria. (Chapter-5).
6. Study immune and stress related genes in aphids after bacterial challenge and characterise the full complementation of the response to bacterial ingestion. (Chapter-5).
7. Identify changes in bacterial gene expression during infection especially those that might lead to aphid mortality. (Chapter-5).
8. Characterise potential virulence genetic elements in *P. poae* by whole genome analysis and gene mutagenesis (Chapter-6).

2 Materials & Methods

2.1 Media

All the media components were of analytical grade and obtained from Difco (Difco Laboratories Ltd, Oxford), Merck (Merckserono, Middlesex, U.K.) or Sigma (Sigma-Aldrich Company Ltd., Dorset, U.K.). Each medium recipe was prepared according to Maniatis *et al.,* (1989). All media shown below were prepared by addition of components to one litre of deionised water. Agar (Difco) was added to the broth medium to a final concentration of 1.5 % (15 g L^{-1}). All media were sterilized by autoclaving at 121 ˚C, 20 kg.m s^{-2} for 20 minutes. Heat labile substances were filter sterilized through a 0.22 µm Millex™ Millipore® filter and added to the media after it had cooled to 50 ˚C. Pre-warmed medium (20 mL) was added to each Petri dish (Thermo Fisher Scientific, Scotland, U.K.).

King's Medium B (KMB) (King *et al.,*1954): Used to differentiate *Pseudomonas* species from one another based on the production of fluorescein. Proteose peptone (Difco) 20 g, K_2HPO_4 1.5 g, $MgSO_4.7H_2O$ 1.5 g, glycerol 10 mL.

Luria Bertani (LB) (Miller, 1972): Standard laboratory nutrient-rich microbiological growth media for the cultivation of many species of bacteria especially *Escherichia coli*. Bacto-Tryptone (Oxoid) 10 g, Bacto-yeast extract (Oxoid) (Oxoid Limited, Hampshire, U.K) 5 g, NaCl (BDH) (BDH laboratory supplies, Dorset, U.K) 10 g, Glucose (BDH) 1 g.

Minimal medium (M9): Contains the minimal constituents for bacterial cells to grow. Carbon and nitrogen sources can be controlled to select for specific phenotypic traits200 mL 5X M9 salts solution; Na_2HPO_4 33.91 g; KH_2PO_4 15 g; NaCl 2.5 g; 2 mL 1M $MgSO_4 \cdot 7H_2O$; 100 µL 1M $CaCl_2 \cdot 6H_2O$; 20 mL 20 % Glucose; 10 mL 100 mg mL^{-1} NH_4Cl.

Super Optimal Broth with Catabolite repression (SOC) (Hanahan, 1983)**:** Nutrient-rich medium enabling optimized recovery of electroporated cells following transformation.

Bacto-Tryptone 20 g; Bacto-yeast extract 5 g; NaCl 0.5 g; 1 M KCl 0.186 g; 20 mM glucose 3.6 g

Ringer's solution ($^1/_4$ strength tablets**,** Sigma-Aldrich Company Ltd., Dorset, U.K.). : Standard isotonic solution used for removing excessive media from bacteria and prevent them from lysis.

Mittler diet (aphid diet): Used in the aphid food sachets. Quantities listed below (Table 2.1) are per 100 mL of water (Dadd *et al.,* 1967).

Table 2.1: Mittler aphid food recipe.		
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-Phenylalanine	40
19	L-Proline	80
20	L-Serine	80
21	L-Threonine	140
22	L-Valine	80
23	L-Ascorbic acid (Vit. C)	100
24	Aneurine Hydrochloride (Vit. 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 (Vit. B6)	2.5
37	D-Biotin - crystalline	0.1

The solution is made by adding each ingredient one at a time to 100 mL of water with 15 g of dissolved sucrose, allowing each component to fully dissolve prior to adding the next. The solution is then measured in aliquots of 25 mL and later stored in plastic tubes in a -20 °C freezer until needed**.**

2.2 Antibiotics

Antibiotics were purchased from Sigma and prepared as described in Table 2.2. Antibiotics were dissolved into the respective solvent at the required concentration and filter sterilized through a 0.22 µm Millex™ Millipore® filter and stored at -20 ˚C.

Table 2.2: Antibiotics and specialized chemicals used in this study.

Antibiotic/Chemical	Solvent	Working concentration
Ampicillin	Water	100 µg mL^{-1}
Kanamycin	Water	50 µg mL^{-1}
Gentamicin	Water	15 µg mL^{-1}
Tetracycline	Methanol	15 µg mL^{-1}
Nitrofurantonin	Dimethylsulphoxide	100 µg mL^{-1}

2.3 Growing conditions for microbes and aphids

Pseudomonas strains & other bacteria isolated from the environment were grown at 27 °C, either on a KB agar plate, or in KB broth overnight with shaking at 200 rpm. All *E. coli* strains used were incubated at 37 °C using LB media either on plate or in broth overnight with shaking at 200 rpm.

All aphid-rearing experiments were carried out using Chinese cabbage (*Brassica napus* L. var *chinensis* cv. Wong Bok) as the host plant. Two different types of aphid rearing were used in the project:

1. **Leaf box rearing**

Each clone was maintained parthenogenetically in the laboratory on excised leaves in small plastic box-cages (Blackman, 1971), at 21 °C, long day (16 h light/8 h dark) regime to ensure no sexual forms were produced. New generations of each clone were set up several times a week by adding four young adult apterae (using a wetted fine paintbrush, size-3) to each box and leaving them to produce about 15 nymphs over a few days. The parents were then removed leaving age-synchronized cohorts of aphids that could then be used to initiate bioassays and subsequent generations when they became adults.

2. **Cage rearing**

The cage rearing was used to generate large aphid populations. As for leaf box rearing, each clone was continued parthenogenetically in the insect cage on 4 weeks old Chinese cabbage pots under 21 °C, long day (16 h light/8 h dark) regime to ensure no sexual forms were produced. New generations of each clone were set up by inoculating plants with aphid populations established for 2 weeks in leaf boxes and leaving them to produce adults up to 4 weeks.

Care was taken when handling aphids to avoid cross-contamination between different clones.

2.4 Bacterial strains, mutants and aphid clones

Table 2.3: All bacterial strains and plasmids used in this project.		
Designation	**Details**	**Source & Reference**
Pseudomonas fluorescens PfR 37 (*P. fluorescens)*	Nitrofurantoin resistant	Isolated from leaf of *Calendula officinalis,* Harris garden, University of Reading, Albertii, 2011
Pseudomonas jessenii PjR8 (*P. jessenii)*	Nitrofurantoin resistant	Isolated from leaf of *Capsicum annuum,* Private garden, Reading, Livermore, 2016
Escherichia fergusonii EfR10 (*E. fergusonii)*	Ampicillin and Nitrofurantoin resistant	Isolated from leaf of *Solanum lycopersicum*, Private garden, Reading, Livermore, 2016
Pseudomonas poae PpR24 *(P. poae)*	Ampicillin and Nitrofurantoin resistant	Isolated from root of *Brassica oleracea,* Experimental green house, University of Reading, Livermore, 2016
Acinetobacter johnsonii AjR35 *(A. johnsonii)*	Ampicillin, Nitrofurantoin and Tetracycline resistant	Isolated from leaf of *Hamamelidae fagale,* Harris garden, University of Reading, Livermore, 2016
Pseudomonas rhizosphaerae PrR91 (*P. rhizosphaerae*)	Nitrofurantoin resistant	Isolated from leaf of *Foeniculum vulgare*, Private garden, Reading, Livermore, 2016
Paenibacillus glucanolyticus PgR18 (Pae. g*lucanolyticus)*	Nitrofurantoin resistant	Isolated from leaf of *Buxus sempervirens,* Private garden, Reading
Escherichia albertii EaR93 (*E. albertii)*	Ampicillin, Nitrofurantoin resistant	Isolated from leaf of *Capsicum annuum*, Cantelo Nursery, Reading, Livermore, 2016
Citrobacter werkmanii CwR94, (*C. werkmanii)*	Ampicillin, Nitrofurantoin and Tetracycline resistant	Isolated from leaf of *Fragaria ananassa,* Experimental green house, University of Reading, Livermore, 2016
Pantoea agglomerans PaR38, (*Pa. agglomerans)*	Nitrofurantoin resistant	Isolated from leaf of *Nasturtium officinale*, Experimental green house, University of Reading, Livermore, 2016
P. syringae pv. tomato DC3000 *(P. syringae)*	Nitrofurantoin resistant	Cuppels, 1983
***Escherichia coli* strains**		
DH5α λpir	*Thi-Pro-Hsd-recA-* zzz::RP4-2 (tet::Mu, kan::*Tn7* [Tp^R, Sm^R])	Simon *et al.,* 1983
DH5α	F– , *recA*, *ΔlacU169(Φ80 lacZΔM15), endA, hsdR, gyrA*	Invitrogen, Life Technologies, USA
Plasmids		
pRK2013	Kan^r, Tra+, ColE1 replicon	Figurski & Helinski, 1979
pBBR1MCS-2	Broad-host-range cloning vector, Kan^r	Kovach *et al.,* 1995
pBBR1MCS-5	Broad-host-range cloning vector, $Gent^r$	Kovach *et al.*, 1995
pK18mobsacB	Allelic exchange suicide vector mobilized by *E. coli* S17-1pir; allows blue-white screening for inserts; Kan^r Suc	Schäfer *et al.,* 1994
pME6010	pACYC177-pVS1 shuttle	Heeb *et al.,* 2000

	vector, Tc^r	
pPR1	*tcaA* gene cloned into broad host range vector pBBR1MCS-2 as Xho I/Eco RI insert, Kan^r	This study
pPR3	*aprX* gene cloned into broad host range vector pBBR1MCS-2 as Xho I/Eco RI insert, Kan^r	This study
pPR4	*tcaA,tcaB &tcaC* genes cloned into broad host range vector pBBR1MCS-2 as Xho I/ BamHI insert, Kan^r	This study
pPR5	*Hypr gene* cloned into broad host range vector pBBR1MCS-2 as Xho I/Eco RI insert, Kan^r	This study
pPM1	tcaA cloned into broad host range vector pME6010 as Xho I/BamHI insert, Tc^r	This study

The Green peach aphid, *Myzus persicae* was used in this work and rest of other *M. persicae* clones, showing different combinations of the insecticide resistance mechanisms are shown in Table 2.4. Clones were originally established from individual ancestral females, collected at different times from widely dispersed populations located in the United Kingdom and mainland Europe. All aphid clones were supplied from Rothamsted research insectary, Harpenden, U.K.

The insecticide resistance mechanisms included in this project:

1. Esterases **(Est)**: Amplified esterase gene EF/FE4, confers resistant to organophosphates and carbamates, moderate cross resistance to pyrethroids.
2. Modified acetylcholinesterase **(MACE)**: Target site mutation in the ace gene, confers resistant to pirimicarb.
3. Knock down resistance mutation **(Kdr)**: (L1014F) of the voltage gated sodium channel, confers 10-30 fold resistance to pyrethroids.
4. Super-knock down resistance **(Skdr)** mutation: (M918T) of the voltage gated sodium channel, confers much higher levels of resistance to pyrethroids than Kdr.
5. **P450**: Amplification of a P450 gene (CYP6CY3) may confer low-moderate resistance to neonicotinoids.
6. **nAChR mut**: Mutation of the nicotinic acetylcholine receptor (R81T), confers very high levels of resistance to neonicotinoids

Table 2.4: Aphid insecticide mechanism clones included in the project.							
Clone	**Country**	**Esterases (Est)**	**Modified acetylcholine sterase (MACE)**	**Knock down resistance (Kdr)**	**Super-knock down resistance (Skdr)**	**Amplification of a P450 gene (P450)**	**Mutation of the nicotinic acetylcholine receptor (nAChR mut)**
4106A	United Kingdom	SS	SS	SS	SS	SS	SS
4225B	United Kingdom	SS	SS	SS	SS	SS	SS
Clone-NS	Germany	SS	SS	SS	SS	SS	SS
794J2	United Kingdom	RR	SS	RR	SS	SS	SS
5191A	Greece	RR	SR	SS	SS	RR	SS*
5444B	Italy	RR	SS	RR	RR	RR	RR
New green	United Kingdom	RR	SR	SR	SR	SS	SS
The above table is based on an allelic discrimination PCR assay(Foster *et al.,* 2000;Bass *et al*., 2011;Field & Foster, 2002;Anstead *et al.,* 2008). ***Key**: SS – homozygote susceptible, SR – heterozygote, RR – homozygote-resistant. *Reduced penetration, there is an evidence that this may be a mechanism that confers low levels of resistance to neonicotinoids in this clone.*							

2.5 Aphid mortality assay

All UK sensitive and resistant aphid bioassays were performed at an insect rearing room at the University of Reading except when using Europe resistant aphid's, which was carried out at the Rothamsted Research insectary. Hence, the general procedure to carry out the aphid bioassays and other physical parameters for rearing aphids, such as leaf box rearing, temperature and humidity, were kept same at both places to minimise chances of variation.

To maintain sterility and avoid contamination, all work was done in a laminar air hood. The aphid mortality assay was designed in four parts:

1. **Preparing the aphid sachets:**

   A perspex plastic cylinder (size 25 mm deep, 25 mm internal diameter, Figure 2.1) was used for preparing aphid sachets. Before use, the plastic cylinders were cleaned with 70 % ethanol and several sets of parafilm sections (4 cm x 4 cm) were prepared. Each set contained 3 square shaped parafilm sections. The first one was covered (stretched) over the upper end of the hollow cylinders and the second section was laid on the plastic tray for sterilization. Both, the hollow cylinder (with parafilm) and the second parafilm section was sterilised in a laminar flow cabinet under U.V light for 30-40 minutes, but no longer than that as over exposure to U.V causes damage to parafilm.

2. Preparing the "Control" sachet

a. Once the parafilm sterilization is complete, the defrosted artificial Mittler diet was filter sterilized using 0.22 µm filter and syringe. Later 600 µL of the filtered diet was aliquoted onto the parafilm stretched over the cylinder.

b. The second parafilm section was then stretched and placed quickly over the diet with the sterilized side being in contact with the diet. Extra care was taken to avoid any spillage and by not touching the centre, as this will contaminate the surface. This sachet was marked as "Control".

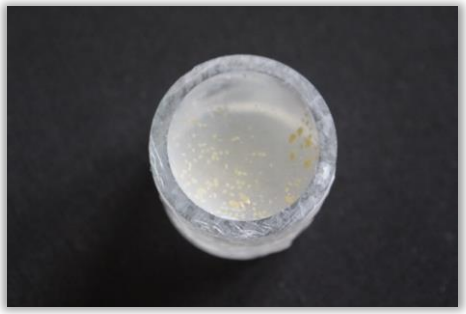


Figure 2.1: Aphids in control sachets

3. Preparing the "Experiment" or "treated" sachets – Inoculating the diet with bacterial strains

a. The bacterial strains were recovered from -80 °C and grown in LB at 27 °C for 12-15 h. The microbial cell density was determined using a spectrophotometer and then normalised to an OD_{600} of 1. This corresponds to a concentration of 10^9 CFU mL^{-1}. The cells were washed in 1 x PBS (1 L of 10 x PBS contains 80 g NaCl, 2 g KH_2PO_4, 29 g $Na_2HPO_4.12H_2O$, 2 g KCl; 1 x PBS has a pH of 7.4).

b. Next the bacterial strains were mixed with the sterile Mittler diet to obtain a final microbial concentration of 10^7 CFU mL^{-1} and then aliquoted onto the parafilm stretched over the cylinder.

c. The second parafilm section was then stretched and placed quickly over the mixture (bacteria + Mittler diet) with the side sterilized being in contact with the diet. Extra care was taken to avoid any spillage and by not touching the centre, as this will contaminate the surface. These sachet were marked as "Experiment or treated" sachets.

d. In this experiment, for each bacterial strain, six concentrations ranging from 10^7 CFU mL^{-1} to 10^2 CFU mL^{-1} along with control aphid sachets were made in triplicate.

4. **Transferring the aphids to sachets**

    a. Ten to fifteen aphids were transferred to each sachet by using a fine, soft paintbrush, to avoid any physical damage to the aphids. The bottom end of the cylinder was covered using another section of Parafilm (non-sterile, as the aphids will not insert their stylus at this end) in order to prevent the aphids from escaping.

Aphid mortality readings were recorded at 24, 48 and 72 h. A bacterium was classed as being pathogenic to the aphids if it triggered aphid death during the 48 h time period. No death was expected in the control sachets.

All mortality readings of all aphid clones after challenge of each bacterium at each dose and time point was tested by two way ANOVA followed by comparison of means by Tukey-Kramer HSD test, using GenStat version 16.0 for Windows (VSN International Ltd, Hemel Hempstead, U.K). In this analysis, "Fit model" tool was used to assess mortality patterns of different aphid clones at various bacterial doses at every 24, 48 and 72 h. Additionally, the probit analysis transforms sigmoid dose-response curve to linear, which can then be analysed by regression either through least squares or maximum likelihood. To calculate LC_{50} values of each bacterium for all aphid clones, 72 h aphid mortality readings at six bacterial concentrations ranging from 10^7 CFU mL^{-1} to 10^2 CFU mL^{-1} were transformed to mortality probits which produced a line of regression. This linear relationship was further imported in GenStat program and by using "Probit analysis tool", logs of explanatory variable (Log concentration of bacteria) and number of responding (Mortality probits) of relationship were analysed. After completion of the analysis, output the provided an effective LC_{50} dose of aphid clone for each bacterium with upper and lower doses at 95 % confidence limits.

2.6 Plant growth conditions

Plants used were Chinese cabbage (*Brassica napus* L. var *chinensis* cv. Wong Bok) (Simply Seed, Nottingham, UK), Organic red sweet pepper Sapporo (RZ) (*Capsicum annum* L.) (Rijk Zwaan UK Ltd, York, UK), sugar beet (*Beta vulgaris*) and *Arabidopsis thaliana* (Col-O ecotype) (Carol Wagstaff lab). Plants were grown in Clover seed modular compost (Clover quality peat product, County Tyrone, North Ireland) containing peat, sand and wetting agents at 75 % humidity, light intensity of 150 µmol m^2 s^{-1} (16 h photoperiod: day temperature of 26 °C, night temperature of 22 °C).

2.7 Bacteria Colonization Assay on Plants

2.7.1 Foliar spray method

All plants were grown in above described compost. Plants were then moved to a growth chamber (22 °C, 75 % humidity, 16/8 h light/dark cycle) three days prior to the assay to acclimatize the physical conditions of the chamber. *P. poae* was grown as described in section 2.3. For each foliar spray the bacteria culture was washed twice with sterile 1 x PBS (1 L of 10 x PBS contains 80 g NaCl, 2 g KH_2PO_4, 29 g $Na_2HPO_4.12H_2O$, 2 g KCl; 1 x PBS has a pH of 7.4) to remove the LB media and re-suspended at an optical density at 600 nm (OD600) of 1.

The bacterial cells suspended in PBS were applied as a foliar spray (both the adaxial and abaxial sides) with a hand atomizer on to the 3 week old plants. Sterile PBS was sprayed onto the un-inoculated, control plants. After spraying they were allowed to dry in sterile flow cabinet. On days 0, 1, 3, 7, 14, 21 and 28, sections (size 0.28 cm^2) of infected leaves and negative control leaves were aseptically removed with a core borer and transferred to sterile micro centrifuge tubes containing 200 µL PBS solution. The leaves were then macerated to slurry using a sterile micro pestle. Seven-fold dilutions (10^2 to 10^{-3}) were made and 10 µL of each dilution was spot plated onto LB agar with Nitrofurantoin in triplicate. The agar plates were left to grow for 16 h at 27 °C and colonies were counted for each sample. The average (from the triplicate samples) was determined and calculated to obtain the CFU per leaf area. The data were transformed (log_{10}) for statistical analysis and graphical presentation, and analysed by ANOVA with the Tukey MCT in GenStat version 16.0 for Windows (VSN International Ltd, Hemel Hempstead, U.K).

The *P. poae* aphid killing efficacy rate was calculated by the formula = (Aphid population on control plants - Aphid population on treated plants)/ Aphid population on control plants * 100.

To facilitate the identity of *P. poae,* colonies were recovered from macerated leaves (without the requirement of presumptive identification) at all-time point sets and polymerase chain reaction was employed to amplify *P. poae* specific TcaA toxin gene (Section 2.14.1).

2.7.2 Leaf infiltration method

Similar to foliar spray, three week old plants were moved to growth chamber (set at 22 °C, 70 % humidity) to acclimatize, prior to infiltration. For each assay the bacterial culture was washed twice with sterile 1 x PBS to remove the LB media and re-suspended at an optical density at 600 nm (OD600) of 1.

A small hole on the underside (abaxial) of the leaf was created by sterile yellow tip. A 1ml sterile syringe (without needle) was pressed against underside of the leaf which infiltrated the bacteria

culture slowly through the small hole. This procedure was applied on other areas of the leaf tissue until 1ml of total bacteria culture was infiltrated into the leaf. 1 mL of sterile PBS was infiltrated for un-inoculated, control plants. The plants were allowed to dry in a sterile flow cabinet. At each time point, the plants were removed from the pots, the leaves were excised and each sample placed into sterile micro centrifuge tubes. They were processed in the same way as the foliar spray method.

2.7.3 Seed soak method

The bacterial suspensions were prepared as described section 2.7.1. In this method, around 10 seeds were soaked in 1 mL of bacteria suspension (10^7 CFU mL^{-1}) for 4 h at 22 °C and then dried for 2 h in a laminar flow cabinet at 22 °C. 1 mL of sterile water was used for un-inoculated, control seeds. After incubation, each seed was sown in individual pot containing sterile clover seed modulator soil. At each time point, the seeds were removed from the pots, the leaves were excised and each sample placed into sterile micro centrifuge tubes. They were processed in the same way as the foliar spray method.

2.8 Aphid Behavioural Bioassay (Olfactometer assay)

This assay was conducted to understand aphid behaviour when they were exposed to different volatile cues of plants (bacterial & water sprayed). The assay comprised into two sections:

1. **Entrainment of volatiles**

In the initial experiment, volatiles were collected from *P. poae* streaked plates directly, with the experimental design described below. Similarly in a second experiment, volatiles were also collected from the three replicates of pepper plants with and without spray bacteria separately. For each entrainment, a single pepper plant was enclosed in a glass vessel, 100 mm diameter and 300 mm in length, open at the bottom and closed with a collection port at the top. The bottom was then closed with two semi-circular aluminium plates that fitted around the bacteria streaked plate/stem of the plant and were clipped to a flange on the open end of the glass vessel. One of the aluminium plates was drilled to accommodate an inlet port, and air, purified by passage through an activated charcoal filter, was pumped into the vessel through this (400 mL min^{-1}). Volatiles were collected on Porapak Q absorbent tubes inserted into the collection ports on the top of the vessels. Further pumps drew air (300 mL min^{-1}) through these tubes. The rates were controlled so that more purified air was pumped in than was drawn out, ensuring that unfiltered air was not drawn into the vessel from outside and obviating the need for a tight seal around the stem, which would have caused damage to the plant. All connections were made with PTFE tubing

and ferrules, and as much as possible the equipment, particularly the glassware, was heated at 180 °C for at least 2 h before use. Porapak Q tubes were conditioned at 140 °C in a stream of purified nitrogen for at least 4 h before use (Blight, 1990). Plants were entrained for 2 days to collect sufficient material for subsequent bioassays. Porapak Q filters were eluted with 0.5 mL of redistilled diethyl ether, and the samples collected were stored in vials in a freezer (−20 °C) for subsequent analysis.

2. Olfactometer Bioassay

A Perspex 4-arm olfactometer, lined on the base with filter paper and lit from above with diffuse uniform lighting was used (Figure 2.2) (Pettersson, 1970). The treated arm inlet tube contained an aliquot (1 µL) of the test solution applied using a micropipette (Drummond "microcaps," Drummond Scientific Co., USA) to a piece of filter paper (4 × 25 mm; solvent allowed to evaporate for one minute). In the control arm, inlet tubes were treated with the same volume of solvent on the filter paper. Air was drawn through the apparatus at 350 mL min^{-1}. Female winged *M. persicae*, obtained from the laboratory culture, were transferred individually from the rearing cage into the central chamber of the olfactometer by using a custom made piece of glass tubing (made from a Pasteur pipette heated over a Bunsen burner to remove the narrower end). Time spent and numbers of entries into each olfactometer arm were recorded with "Olfa" software (F. Nazzi, Udine, Italy) over a 16 min bioassay period during which the olfactometer was rotated through 90° every 2 mins to avoid directional effects. Mean time spent in and number of entries into treated and control arms were compared using a paired t test (GenStat, 16th Edition).

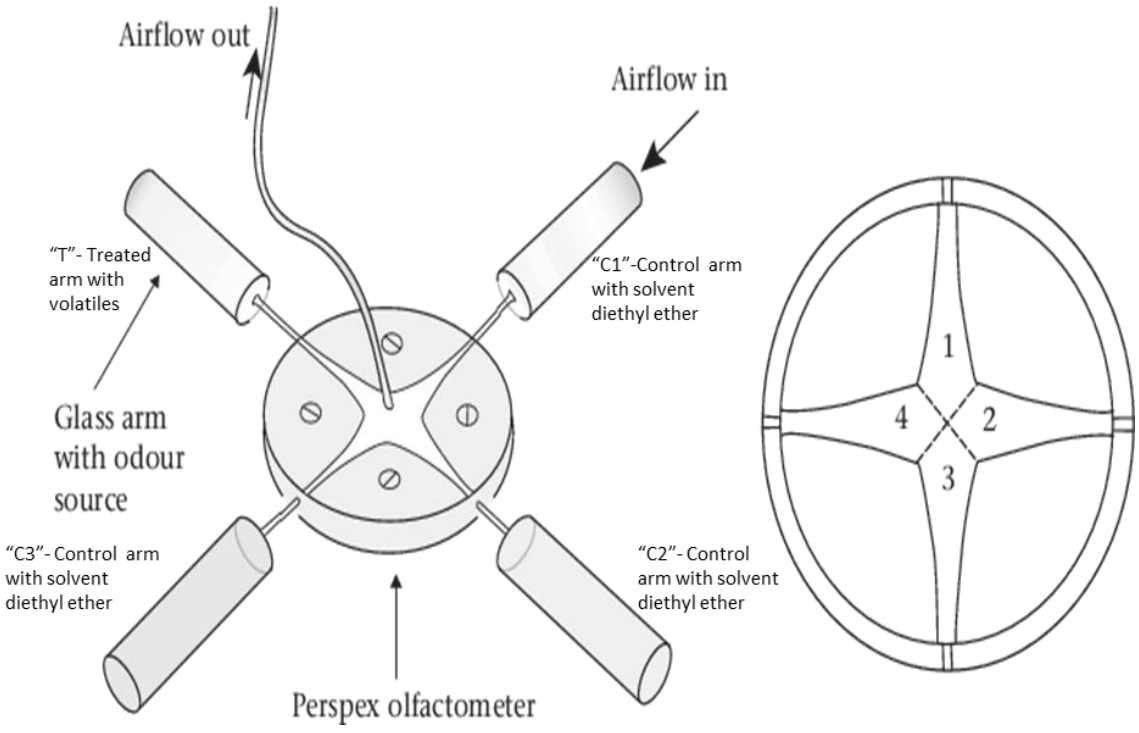


Figure 2.2: Diagrammatic representation of the four-arm olfactometer with cylindrical glass arms used to contain odour sources alongside diagram showing division of regions within the olfactometer. Figure designed and published by (Webster *et al.*, 2010)

2.9 Aphid Behavioural Bioassay – Choice and No choice experiment

Ten pepper plants were moved to a growth chamber (22° ˚C, 75 % humidity, 16/8 h light/dark cycle) to acclimatize for three days prior to the assay. The bacterial suspensions were prepared as described in section 2.7.1. The bacterial cells suspended in phosphate buffer were applied as foliar spray (both the adaxial and abaxial sides) with a hand atomizer on to the 3 weeks old plants. Sterile PBS was sprayed onto the un-inoculated, control plants. After spraying they were allowed to dry in sterile flow cabinet.

In choice experiment, both inoculated and control plants were then placed inside a 60cm^2 bug dorm aphid tent (Watkins & Doncaster, Leominster, U.K). 40-50 starved aphids were then introduced in to the centre of the tent and left to migrate to any of the plants. Whereas in No-choice experiment, pepper plants sprayed with bacteria and sterile water were placed in two separate tents and 25-30 starved aphids placed in the each tent.

In both cases, the numbers of aphids on each plant were counted 1, 2, 3, 7, 14 and 21 days after introducing the aphids.

2.10 Standard Bacteria Growth curve

For growth rate assay, bacterial strains were grown overnight at 27 °C and microbial cell density was normalised to an OD_{600} of 1 which was further diluted by a factor of 1:100. Then, 10 µL of each dilution was added to a 100-well microplate containing 90 µL of appropriate media per well. Optical density at 600 nm was measured every 20 min at an incubation temperature of 20 °C, with 20 sec shaking before reading for 24 h using a microplate spectrophotometer (BIOSCREEN C, Growth curves, USA). The time to reach an absorbance reading of 0.0825 arbitrary units (approximately three times above the blank signal) was determined and used to plot calibration curves for this assay. Vmax (measured as milli-optical density units per minute (mOD min^{-1}), the maximal rate of change in optical density during log growth, was calculated on exponential phase of growth cycle.

2.11 Bacterial quantification inside aphids

To quantify bacteria within aphids, control and bacteria inoculated aphid sachets (described in aphid mortality assay) were prepared in four replicates for 24, 48 and 72 h. Ten aphids (from both control and treated sachets) were surface sterilized by 10 % sodium hypochlorite for 5 min and

washed 3 times with sterile water. Next, the pooled ten aphids from non-inoculated and inoculated sachets were homogenized by sterilized pestle and suspended in 200 µL of sterile Phosphate Buffer Saline (PBS). A dilution series was prepared per sachet and aliquots plated onto LB agar with Nitrofurantoin. Plates were incubated overnight at 27 ˚C and colonies counted the next day and calculated to give CFUs per aphid. The data were transformed (log_{10}) for statistical analysis and graphical presentation, and analysed by ANOVA (GenStat, 16th Edition).

2.12 Protein estimation in bacteria filtrate by Bradford assay

To quantify soluble protein in bacteria filtrates, bacteria were grown in two different media - Mittler diet and LB media for 18, 24, 36, 48, & 72 h at 20 ˚C growth conditions. The cells were separated from the media by filtration and the filtrate of the diet was used to test amount of protein. The concentration of soluble protein in all filtrates was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (Merck-Schuchardt, Hohenbrunn, Germany) as the standard.

2.13 Extraction and purification of nucleic acids (DNA)

Centrifugation was performed using a table top Heraeus Sepatech Biofuge 13 centrifuge. Procedures, following the manufacturer's instructions supplied with the various kits, were carried out in Eppendorf test tubes at 13,793 g unless otherwise stated. DNA was stored at -20 ˚C until required.

2.13.1 Plasmid miniprep

Plasmid DNA minipreps were carried out using a Qiagen mini prep kit. Dependent on the plasmid copy number, 1.5 mL to 5 mL of an overnight culture was used to extract DNA. In the final step, DNA was eluted into either 30 µLl of the supplied EB buffer or sterile water as required.

2.13.2 Total chromosomal DNA extraction

Chromosomal DNA was extracted and purified using a Qiagen DNeasy® Blood and Tissue kit. Following the protocol guidelines for Gram-negative bacteria pre-treatment, an *E. coli* culture was grown to 0.5 OD and cells were harvested by centrifuging at 8,000 g for 10 mins. Pre-treated cell pellets were further processed according to the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" and the DNA were eluted in 100 µL of elution buffer. A sample

was electrophoresed in a 0.8 % agarose TBE gel to check for DNA integrity and the concentration was measured using a NanoDrop® ND-1000 UV-Vis Spectrophotometer.

2.14 Polymerase Chain Reaction (PCR) set up and cycling conditions

All PCR reactions were carried out in a table-top Techne Thermal Cycler. Different polymerases and PCR mix were used for the different PCR tests carried out: PCRBIO Taq Mix Red conditions were used for standard PCR and High fidelity PCRs requiring proof-reading enzymes for all cloning steps. Cycling conditions for all polymerases and mixes are detailed below.

2.14.1 PCRBIO Taq Mix Red PCR conditions

Standard PCRs were performed using PCRBIO Taq Mix Red (PCR Biosystems Ltd, London, U.K.). Taq Mix Red was used to set up a PCR in the following protocol: 2x PCRBIO Taq Mix Red; 1 µL of each 10 µM forward and reverse primer; 1-2 µL template (~100 ng genomic or plasmid DNA); molecular biology grade water to 25 µL. Cycling conditions were as follows: initial denaturation at 95 °C for two mins; 30 cycles of denaturation at 95 °C for one minute, annealing at 50–65 °C for 20 seconds – 1 minute (depending on primer pair used) and extension at 72 °C at one minute kb^{-1}; and a final extension step of 72 °C for five minutes.

2.14.2 Phusion PCR conditions

Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Scotland, and U.K.) buffers and enzyme were used as required for the generation of constructs, mutants and sequencing. The reaction was prepared as follows: 10 µL 5x Phusion HF buffer; 1 µL 10 mM dNTPS; 1 µL of each 10 µM forward and reverse primer; 0.5 – 1 µL template; 0.5 µL Phusion polymerase (1 unit/50 µL); molecular biology water to 50 µL.

Cycling conditions were as follows: initial denaturation of 98 °C for 30 seconds; 30 cycles of 98 °C for ten seconds, 50–70 °C (dependent on primers used) for 30 seconds, 72 °C at 30 seconds kb^{-1}; and a final extension of 72 °C for ten minutes.

A list of primers used in this study can be found in Table 2.5. All primers were designed using Primer3Plus (Untergasser *et al*., 2012), checked for their specificity using NCBI Primer-BLAST (Ye *et al*., 2012) and any hairpins, dimers and cross-dimers were predicted using Netprimer (PremierBiosoft).

All primers for qPCR were designed in the same manner as above, but with the following parameters: 100-200 base pair (bp) product size; paired towards 3’ end; GC content of 50-60 %;

optimum melting temperature (Tm) of 60 °C. All primers used in this work were obtained from Eurofins MWG Operon (Eurofins Genomics, Ebensburg, Germany).

Table 2.5: Primers used in this study, subcategorised into diagnostic, cloning, and qPCR primers.						
Target	**Primer Name**	**Sequence**	**Tm (°C)**	**Product Size (bp)**	**Application**	**Reference**
Diagnostic Primers *tcaA* toxin	TcaA1poae_F1 TcaA1poae_R1	TAAGGATTACACCGGCCAAC TTTCTTTCAACGGCTGCATT	58	524	*P. poae* diagnostic primers	This study
aprX toxin	Poae_aprA_F4 Poae_aprA_R4	CCCGGACCTGAACAACTATG GCACCGTACACGAATGTATC	60	700	*P. poae* diagnostic primers	This study
rhs4a toxin	Rhs_F3 Rhs_R3	CACCACACGCTATGACTATG CTCCTGCAACAGACGTAAAC	60	1300	*P. poae* diagnostic primers	This study
Deletion of *tcaA* gene	5'deltatcaA_F 3'deltatcaA_R	CTGACCCAGTTAAGCGAATC GGCGAAGCTGTCTTTATCAC	57	1109 (Deletion mutant) 3.9kb (Wild type)	*tcaA* gene deletion diagnostic primers	This study
Deletion of *tcAB* gene	5'deltatcAB_F 3'deltatcAB_R	GCAACACCGGCTGTCTACTC GCTCAAAGCCGACCATGAAC	60	1202 (Deletion mutant) 10kb (Wild type)	*tcAB* gene deletion diagnostic primers	This study
Deletion of Hypr gene	5'delta hypr F 3'delta hypr R	GCGTCGGTCACTTGTACTTG AGGTGGTGATGAAGGTTTCG	60	1470 (Deletion mutant) 2658 (Wild type)	*Hypr* gene deletion diagnostic primers	This study
Deletion of *rhs4a* gene	delta RhsA2 F delta RhsA2 R	TCAACGAAGCCCAATTCACC CTGGCATTGAACGAGTTGTC	57	1796 (Deletion mutant) 5204(Wild type)	*rhs4a* gene deletion diagnostic primers	This study
Deletion of *aprX* gene	delta IP F delta IP R	CGGTGGTCATGGAAACCTAC GAATAAGTCCCGCGACCCAC	62	1647 (Deletion mutant) 3099 (Wild type)	*aprX* gene deletion diagnostic primers	This study
Cloning Primers 5' arm *tcaA* toxin	5'tcaA_F 5'tcaA_R	AAAggatccACCCTGCATACCCAACTCGAAGAAG CGCTCTTGCGGCCGCTTGGAACGGTCGCTGAGTG AGCGAGGAGAAATC	58	479	Amplification of 5'arm of *tcaA gene*	This study
3' arm *tcaA* toxin	3'tcaA_F 3'tcaA_R	CCGTTCCAAGCGGCCGCAAGAGCGCGGTTCAAGG AATGACCATGTCTG ATTgaattcTTCGAAGCCGCTTAAGTAACCGTG	58	534	Amplification of 3'arm of *tcaA* gene	This study
3' arm *tc* toxin	3'tcaBC_F 3'tcaBC _R	CCGTTCCAAGCGGCCGCAAGAGCGCATTAACGGC GGTGAGGGTTCATC ATTgaattcTATGGCTACATTGGCCCAACCAAG	58	581	Amplification of 3'arm of *tc* gene	This study
5' arm Hypothetical protein	5'hypr F 5'hypr R	AAAggatccCACGGGTGTCGAACACGAAACTGG CGCTCTTGCGGCCGCTTGGAACGGGCCTTGCAGG CGTTTCACAGGATG	59	557	Amplification of 5'arm of *hypr* gene	This study
3' arm Hypothetical protein	3'hypr F 3'hypr R	CCGTTCCAAGCGGCCGCAAGAGCGCTGGCATCGA GCTGATGACTAGG ATTgaattcACTCATGCGCTCGACACAGCGTTC	59	491	Amplification of 3'arm of *hypr* gene	This study
5' arm *rhsA2*	5'RhsA2 F 5'RhsA2 R	AAAggatccGGCGAGAGCCTGCAAAGCAATTTC CGCTCTTGCGGCCGCTTGGAACGGCATAGGCGGC CACTTCCAGTACAC	58	453	Amplification of 5'arm of *rhs4a* gene	This study
3' arm *rhsA2*	3'RhsA2 F	CCGTTCCAAGCGGCCGCAAGAGCGATCATTGGGA	58	458	Amplification of	This study

	3'RhsA2 R	CACCTGGAGGTATC ATTgaattcGAAGCCTGAAGCCTGAAAGTCAAC			3'arm of *rhs4a* gene	
5' arm *aprX*	5'IP F 5'IP R	AAAaagctt CCGGCTTGCAGATGGTCAACAATG CGCTCTTGCGGCCGCTTGGAACGGACGCAGGTTC AAGTTGCAGAAGTC	59	643	Amplification of 5'arm of *aprx* gene	This study
3' arm *aprX*	3'IP F 3'IP R	CCGTTCCAAGCGGCCGCAAGAGCCCGGCGATGCT GTACTGAGCTATG ATTgaattcCGGGCTTGATAGTCGCCCTCTTTC	57	508	Amplification of 3'arm of *aprx* gene	This study
tc toxin	Tc_Comp_F1 Tc_Comp_R2	AAActcgagCCGAGATGGAAGCGATGAAG ATTggatccAACCCTCACCGCCGTTAATG	62	12kb	Amplification of whole *tc gene*	This study
tcaA toxin	Tca_Pro_F1 Tca_Pro_R1	AAAaagcttCCGAGATGGAAGCGATGAAG ATTgaattcTCAGGCAGTACGCTATTGAG	60	3kb	Amplification of whole *tcaA gene*	This study
Hypothetical Protein	Hyp_Comp_F1 Hyp_Comp_R1	AAActcgagCTTTGCGGCGGGTGAATAAC ATTgaattcGCCTAGAGCCTAGTCATCAG	60	1304	Amplification of whole *hypr* gene	This study
rhs4a toxin	RhsA2 comp_F Rhs_comp_R1	AAActcgagGGCGAGAGCCTGCAAAGCAATTTC TTTgaattcGCCTGACAGTACGAATGATG	61	4735	Amplification of whole *rhs4a* gene with promoter region	This study
aprX toxin	IP_Comp_F1 IP_Comp_R1	AAActcgagGCGAGGTTCCTATCGAACAAAGACTG TTTgaattcGCGGAGGTGGGTAATACAAGAC	61	1637	Amplification of whole *aprX* gene	This study
aprX toxin	IP_Pro_F1 IP_Pro_R1	AAAgaattcCCGGCTTGCAGATGGTCAACAATG ATTctcgagCGGGCTTGATAGTCGCCCTCTTTC	61	2372	Amplification of whole *aprX* gene with promoter	This study
qPCR reference gene primers *rpoS*	Poae_rpoSF1 Poae_rpoSR1	AAGTGCCGGAGTTTGACATCG GTGCGAACTGAAGGTGGTGAT	61	106	Housekeeping gene for qPCR (bacteria)	This study
rpoD	Poae_rpoDF Poae_rpoDR	GGAAAAGCGCAACAGCAGTC GGCGCGACTTCGAATACGTT	61		Housekeeping gene for qPCR (bacteria)	This study
actin	Mp_ActF1 Mp_ActR1	GGTGTCTCACACACAGTGCC CGGCGGTGGTGGTGAAGCTG	61	100	Housekeeping gene for qPCR (aphid)	This study
aph	Mp_Aph1R Mp_Aph18	TGGTATACACGTTGGTTCTC GACCACGAGCTTCCCCGTG	61	100	Housekeeping gene for qPCR (aphid)	This study
qPCR *M. persicae* gene primers Venom protease	Mp_VP_F1 Mp_VP_R1 Mp_VP_F2 Mp_VP_R2 Mp_VP_F3	GCAGGCGTATTCTTGTCAGC GAGACACTTGCTCGGGTGAT CAGCGTTTCGTCGGGAAAAC CGTCGAACTCTCCGAAGCAG GCCCAACGAGCAAAATTCCC	61 61 61	124 162 144	qPCR for gene expression levels	This study

	Mp_VP_R3	GTTTCCACACATGGCGCTAA				
Cathepsin B-N	Mp_cathepsin_F1	ACATGGAAGGCAGGTGTGAA	61	177	qPCR for gene expression levels	This study
	Mp_cathepsin_R1	TCTCCTGGCGTCAAAGTGTC				
	Mp_cathepsin_F2	AGGCAACTGTGGATCGTGTT	61	149		
	Mp_cathepsin_R2	CTCCATCACACCCAAAGCCA				
	Mp_cathepsin_F3	CAGGGCAATAACACTTGCGC	61	182		
	Mp_cathepsin_R3	ACGATGCTTCAATGGGTCCG				
Noggin	Mp_Ng_F1	ACGTACAGTCTTCAGTCACAGG	61	129	qPCR for gene expression levels	This study
	Mp_Ng_R1	TCAACAAGACGAGAGGGCAC				
	Mp_Ng_F3	AAATGACTTGACGCCAGCCA	61	119		
	Mp_Ng_R3	TCGACAAGCTCAACCGACTC				
	Mp_Ng_F4	TCTTCTTGGCGGGTGAGTTG	61	188		
	Mp_Ng_R4	TGACCTGCTCGGTACAAGAC				
Larval cuticle	Mp_Cuticle_F2	GTCAGAGTCCAGCAACCTGT	61	105	qPCR for gene expression levels	This study
	Mp_Cuticle_R2	TTTGGATACGGCGTGAACGA				
	Mp_Cuticle_F3	GATGTCACCACTCGACGGAT	61	113		
	Mp_Cuticle_R3	TCACAAGTTCAGCGTTCCTGT				
Alpha-tocopherol	MP_Toco_F2	CACCCCGCCGAGTATTTGAG		102	qPCR for gene expression levels	This study
	MP_Toco_R2	TGGAACACCAACGAGTTCGA				
	MP_Toco_F3	TTCGTCCATCCTGTGTGGTG		136		
	MP_Toco_R3	AGAGTTACCGGACTACGTGG				
Cytochrome P450 6a13	Mp_cycP450_F1	TTCTTCCGCTTGATCTTTCCG	61	180	qPCR for gene expression levels	This study
	Mp_cycP450_R1	CACCATAATAGCGACATTAACGAGA				
	Mp_cycP450_F2	CGGAAACAGGATTCCGAGTAGG	61	148		
	Mp_cycP450_R2	CGACGTCATTGGAACTTGCG				
Carotenoid desaturase	Mp_CAT_F1	GAGTTGGTGGTACAGCAGCA	61	192	qPCR for gene expression levels	This study
	Mp_CAT_R1	TGTCCTCCCCCAAATCTTCG				
	Mp_CAT_F2	ACTATGTACTTGGGAATGTCACCAT	61	213		
	Mp_CAT_R2	TCCTTTCGCCACTCCTTTGT				
	Mp_CAT_F3	ACGATCCGAGATCATGGCAA	61	159		
	Mp_CAT_R3	CAGTACCAGGGTGTGCAGAA				
Gammaglutamyltranspeptidase	Mp_ggt_F1	GCCGATGCTGCTGAACATTT	61	250	qPCR for gene expression levels	This study
	Mp_ggt_R1	ACTTTGTTCTTGGAGCGGGA				
	Mp_ggt_F3	CTCCGCCACCGTACAAGTTA	61	229		
	Mp_ggt_R3	GCCGTTTGTCCACAGCAAAT				
	Mp_ggt_F4	GGTGGCGGTCCCAAATGTA	61	239		
	Mp_ggt_R4	TTGAAGTCAGCCGGGGATAC				
Olfactory receptor	Mp_OF_F1	ATTCTGGACTTGCCGTGTTG	61	140	qPCR for gene	This study

	Mp_OF_R1	AGTTACCACGCCAGCAGC			expression levels	
	Mp_OF_F2	TGGTGAAAGCGTATGGAAGGT		143		
	Mp_OF_R2	ACTGCAAGACAAAAGCCACA				
	Mp_OF_F4	TTTGCATCAGTTCTTGGAGCT		116		
	Mp_OF_R4	AAATGCCACATGCTTGGTGA				
Major facilitator superfamily domain-containing 6-like	Mp_mfs_F2	CACGGCCTCCAAAACACAAA	61	198	qPCR for gene expression levels	This study
	Mp_mfs_R2	CCACGACCAATTGATCCCCA				
	Mp_mfs_F3	TACCACCCGGAAACGAAACC		166		
	Mp_mfs_R3	ATCTAACGGCGAACCCAACA				
	Mp_mfs_F4	GAACGCTCCAAGCTCTCCTT		137		
	Mp_mfs_R4	TATCGCTAACACAGCACCGC				
Facilitated trehalose transporter Tret1-like	Mp_Tre_F2	CCAATACTTGTGTGCCCGGT	60	120	qPCR for gene expression levels	This study
	Mp_Tre_R2	CGCGAGTACCAGCCAACTTA				
	Mp_Tre_F3	GTGCTGCTGTTCTACTCGGT		222		
	Mp_Tre_R3	GGCCACGTCCTTGTAAATGC				
	Mp_Tre_F4	GATGGCCGTCAAAGGGGTAA		176		
	Mp_Tre_R4	CTGGCATTATGAACGCACCG				
legumain	Mp_lg_F2	AAATCCCGAACCTGGTGTGA	60	212	qPCR for gene expression levels	This study
	Mp_lg_R2	CTCCAAGACCCGTGTGGAAA				
	Mp_lg_F3	GTCATTCTGGCGCCATGTTC		193		
	Mp_lg_R3	CTCTTCCTCAATCCGTCGGG				
Lycopene	Mp_lyco_F1	ATCATTTCCCAGAGCCCTCG	60	125	qPCR for gene expression levels	This study
	Mp_lyco_R1	GGGATCTCGTCTTCGACAGC				
	Mp_lyco_F2	ACATCGCACCGAAACTTCCT		204		
	Mp_lyco_R2	GGACCCCATACGAAGCAGAG				
qPCR *P. poae* gene primers AprX-Serine protease	Poae_aprA_F1	AGTCAATGGCAAACCGTCCT	60	195	qPCR for gene expression levels	This study
	Poae_aprA_R1	GTGCTTTCTGCTGGGTGTTG				
	Poae_aprA_F2	AGAAGCTCTACGGTGCCAAC		164		
	Poae_aprA_R2	GAAACCGGAGAAGTCCAGGG				
	Poae_aprA_F3	ATTCGTGTACGGTGCCAGTT		176		
	Poae_aprA_R3	ACCCGAGGCATAGCTCAGTA				
PvdD-NRPS	Poae_pvdD_F1	GTAGTGGATTACCTGGGGCG	60	153	qPCR for gene expression levels	This study
	Poae_pvdD_R1	CACCAGTTGTTTGCCTGTCG				
	Poae_pvdD_F2	CGATGTTCGGTGAAGCAACC		155		
	Poae_pvdD_R2	CAAGCCATCCAACTGGTCCT				
	Poae_pvdD_F3	ACACACGCCACTGTTTGAGA		169		
	Poae_pvdD_R3	TGGATAGTGGGTCTGCTCCA				
	Poae_pvdD_F4	GAACAGCAAATGGCAACGGT		117		

	Poae_pvdD_R4	CGCCTCTACCAAACTGCTGA				
PvdF-synthetase (Pyoverdine biosynthesis)	Poae_pvdF_F1 Poae_pvdF_R1 Poae_pvdF_F2 Poae_pvdF_R2 Poae_pvdF_F3 Poae_pvdF_R3	CCTGGTGTATGTCTGGTCCC CCAGAAACTCCAGCACCGAT ACCTGGAATGCGCTATACGG ATCTCGGTCTTGAGCACGTC CGGACTACGGCTTCGCTTAT GACGCTCGAAATCCTGCTTG	60	107 164 168 	qPCR for gene expression levels	This study
EfeoB1 (peroxidase)	Poae_EfeB_F1 Poae_EfeB_R1 Poae_EfeB_F2 Poae_EfeB_R2	GCATGGTCGGTTTTCCCAAC CAGGGCGTGGATATTGGTGT GGCGTCAACAAAAACGGTCA GAAGAAATACCCGCCACCCA	60	104 150 	qPCR for gene expression levels	This study
Thymine DNA-glycosylase	Poae_mug_F1 Poae_mug_R1 Poae_mug_F2 Poae_mug_R2	CTTGCATGTCCTGTTCTGCG CAAGGTGGTCAATCCGCAAC TCGAACACAAGATCCGCCG ATTGATCGAGGCTGAACGCC	60	181 183 	qPCR for gene expression levels	This study
AHYP -Alkyl hydroperoxide reductase	Poae_AHYP_F1 Poae_AHYP_R1 Poae_AHYP_F2 Poae_AHYP_R2	GGTCAACTTCCCGATCCTGG GATAGGTGATGGTCAGGCGG GATTCCCTGCAACTGACCGA TTGGGTTGTGGGGTAAGACG	60	146 170 	qPCR for gene expression levels	This study
RND efflux membrane fusion protein	Poae_RND_F1 Poae_RND_R1 Poae_RND_F2 Poae_RND_R2	GTGTCGGTGGAGTTGTGGAG CCGATAGGGGAATGACACCG CTTTCCTGCCCCTTGCTTTG ACCTCACCTGGGTAGCTGTC	60	190 148 	qPCR for gene expression levels	This study
Fimbriae usher protein StfC	Poae_fimbriae_F1 Poae_fimbriae_R1 Poae_fimbriae_F2 Poae_fimbriae_R2	CAAGGGCGTACTGACAACCT CAGGTTTGAACGCAACGAGG ATGACGGTGCCTACTTGTCG CATGGGCGATAAAACCGCTG	60	180 192 	qPCR for gene expression levels	This study
Arginine deaminase	Poae_arcA_F1 Poae_arcA_R1 Poae_arcA_F2 Poae_arcA_R2 Poae_arcA_F3 Poae_arcA_R3	CGCAAAGTCATGGTGTGCTC TGGTGACGAAGTCGAAGTGG ACGCTCAACCCGATGTACTG CGATCAACACCACGCCATTG CGCAACACCTACACCAACAC TAGTCGATAGGGTCGCGGAT	60	134 190 130 	qPCR for gene expression levels	This study
Haem oxygenase	Poae_HOX_F1 Poae_HOX_R1 Poae_HOX_F2 Poae_HOX_R2	CGCCTGAACCAGATCACCAA TACAGGGCTACCAGTTCCGA TGGGCCTGAGTGAAACCTTC AAGCGCACAAACGCATCAAT	60	140 160 	qPCR for gene expression levels	This study
Hypothetical	Poae_Hyp_F1	CCTAGTCAGCAAGGCGTCG	60	119	qPCR for gene	This study

protein-toxin	Poae_Hyp_R1 Poae_Hyp_F2 Poae_Hyp_R2 Poae_Hyp_F3 Poae_Hyp_R3	ACAAGGTATGCCAGTGAGGC ACCTTGTTCGACATCTGCCC GATGGTAGGTCCAGTCCAGC ACGTATGCCGCTGGTTACTG TCGAACAGCGTCGAATCCC		 121 157 	expression levels	
TcaA toxin	Poae_TcaAF Poae_TcaAR TcaA1poae_F2 TcaA1poae_R2	CGTTCCCTTGATGAGGTGCT GAGGCTGATGCGGTAACTGA CACTACGGAGCTGCACAAAA AGTGCGGATGGAGAACAAAC	60	135 110 	qPCR for gene expression levels	This study
TccC toxin	TccC1poae_F1 TccC1poae_R1	CGAGTGGTTGAATGTGTTGC TCTCGTCCAACTCCAGGGTA		153	qPCR for gene expression levels	This study
Restriction enzymes sites are described in lowercase and underlined letters						

2.15 Agarose gel electrophoresis

Gels were self-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 Ambion® TBE 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 min - 1 h). BIOLINE HyperLadder™ 1 was most often run in tandem with the samples as a DNA band size marker. On completion of the run, DNA bands were visualized under UV-light and photographed using POLAROID film.

2.16 PCR purification

PCR products were purified using the Genomic DNA Clean & Concentrator™-25 kit (Zymo Research, Irvine, U.S.A) according to manufacturer's instructions. The purified PCR products were eluted in 25 µL using ultra-pure water and stored at -20 °C.

2.17 DNA gel recovery

DNA extraction from gel was carried out using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, USA). Target DNA bands were excised from gels using sterile scalpels and weighed. Samples were placed into clean 1.5 mL Eppendorf tubes together with 3 volumes of ADB Buffer (i.e. 300 µL of buffer was added to a 100 µg sample). Samples were placed into a hot water bath and incubated at 55 °C for 10 minutes, or until completely dissolved. The melted agarose solution was transferred into a Zymo-Spin™ Column which was placed into a clean 1.5 mL collection tube. The sample was centrifuged at maximum speed for 10 seconds. The flow-through was discarded and 200 µL of wash buffer was added to the column which was spun for 10 seconds. The wash stage was repeated with 200 µL of wash buffer and spun for 30 seconds. The Zymo-Spin™ Column was placed into a new 1.5 mL collection tube. Directly to the centre of the column matrix was added 25 µL of sterile water. The column was spun for 1 minute to elute the DNA. DNA concentration was measured on a NanoDrop® ND-1000 UV-Vis Spectrophotometer.

2.18 Restriction digestion

PCR products and plasmids (100-500 ng) were combined with restriction enzyme (1-5 units) in the presence of 1X enzyme-specific buffer and the reaction volume was adjusted to 20 µL with ultra-pure water. Reaction mixtures were then incubated at 37 °C water bath for 5 - 120 mins (dependent upon manufacturer's recommendations). In cases of simultaneous digestion by two restriction enzymes, 1 unit of each enzyme was used and an appropriate buffer was selected to ensure maximum enzyme activity.

2.19 Preparation of electro competent cells and electroporation

A single colony from an agar plate, with specific antibiotics or growth requirements, was selected and grown overnight in liquid medium. From this overnight culture, 1 mL was transferred to an Erlenmeyer flask containing 100 mL of fresh LB or KB broth. The culture was incubated at 37 °C on a rotary shaker (225 rpm) and OD600 measurements were taken on an Eppendorf BioPhotometer™ until this reached between 0.3-0.4 (usually 2-3 h). The culture was then immediately placed into an ice-bath slurry and mixed by hand for 2 mins to allow for an overall balanced cooling and then left for a further 30 mins. The culture was aseptically distributed into pre-chilled and sterile 50 mL falcon tubes and cells were collected by centrifugation at 3000 g for 10 mins in a Sorvall Instruments RC-3B™ refrigerated centrifuge set at 4 °C. The supernatant was discarded and the cells were washed 3 times with an ice-cold sterile 20 % glycerol solution. This procedure serves to remove the binding of salts from the cells which can greatly lower the efficiency of electroporation and cause arcing. After the final wash, all cells were amended with 2 mL of sterile ice-cold 20 % glycerol solution. Aliquots (50 µL) were distributed into sterile collection tubes and placed into liquid nitrogen (1 min) before storage at -80 °C.

As above, template DNA was incubated with aliquots (50 µL) of competent cells on ice for 30 mins. Cells were added to pre-chilled 1 mm BIO-RAD Gene Pulser® cuvettes and lightly tapped to evenly distribute the cells. The cuvette was placed into the apparatus and electroporation was performed at 1.8 kV, 25 µF with the pulse controller set to 200 Ω. The pulse was applied by pressing both buttons simultaneously. The cuvette was removed and immediately amended with 1 mL of SOC medium. Cells were transferred to a 1.5 mL sterile tube and left to recover by incubation at 37 °C with shaking at 225 rpm. Aliquots of the transformation culture were plated onto LB plates supplemented with the appropriate antibiotics and other growth requirements as needed.

The procedure was modified for preparing electro competent cells of *Pseudomonas* i.e. 1.5 mL overnight culture was washed 3 times with ice-cold sterile 0.5 M sucrose. After the final wash, all

cells were suspended in 50 µL of ice-cold sterile 0.5 M sucrose. The DNA (~10 µL) was incubated with aliquots (50 µL) of competent cells on ice for 30 mins, followed by electroporation at 2.5 kV, 25 µF with the pulse controller set to 200 Ω. Cells were immediately recovered by adding 1 mL KB and the cells incubated at 27 °C with shaking at 225 rpm for minimum 3 h. Aliquots of the transformation culture were plated onto LB plates supplemented with the appropriate antibiotics and other growth requirements as needed.

2.20 Conjugation – triparental mating

In this study *E. coli* DH5α was routinely used as a donor. This strain does not have the mobilisation functions required to enable transfer of plasmids to recipient strains. A helper strain, HB101, containing a plasmid (pRK2013) expressing the required transfer proteins must therefore be used to ensure successful conjugation. *E. coli* donor, pRK2013 and the *P. poae* recipient were grown overnight. 0.5 mL of helper and donor, and 1 mL of recipient, cells were centrifuged for 1 min at 1,300 g and washed twice with ¼-strength Ringer's solution. The cells were finally re-suspended the last time in 500 µL of ¼-strength, mixed together and centrifuged for 1 min. The supernatant was discarded and the pellet was plated onto KB agar and incubated for 24-48 h at 30 °C. The cells were streaked out on selective media.

2.21 General Cloning procedure

The purified PCR products and appropriate cloning vector were quantified as described above (in section 2.15) and digested with the appropriate restriction enzyme(s) before use in a ligation reaction. Insert: vector ratios of 3:1 and 5:1 were used and the quantity of PCR product to be added to the molar mix was calculated with the following equation:

(ng of vector x kb size of insert) x (insert: vector molar ratio) = ng of insert

The digested PCR product was mixed with digested vector, 10X ligation buffer (1 µL) and T4 ligase (3 units). The reaction volume was adjusted to 50 µL with ultra-pure water. The components were mixed by pipetting and left to incubate at room temperature for one h. After 1 h they were transformed into competent cells and spread onto LB containing the appropriate antibiotics (as described in section 2.2).

2.22 Gene knockout mutagenesis and complementation

Selected Toxin genes (*tcAB, tcaA, rhs4A, aprX, hypr*) were knocked out using the allelic exchange method (Merlin, *et al.*, 2002). N (N-terminal) and C (C-terminal) primers anneal upstream and downstream of the target gene, respectively, while "i" (inside) and "o" (outside) indicate whether the priming site is closer to or further from the target gene. The Ni and Ci inside primers are designed to leave the ends of the targeted genes intact in order to retain the original translational signals in the final construct. In addition, Ni and Ci contain 24-nucleotide-long 5'tails with complementary sequences. Briefly, ~500 bp regions either 5' or 3' to the gene(s) of interest were amplified by PCR with the appropriate Ni/No and Ci/Co primers for each selected target, using Phusion polymerase and reaction conditions (see Phusion PCR conditions). PCR products were purified using Genomic DNA Clean & Concentrator™-25 kit (Zymo Research, Irvine, U.S.A). The primers contained restriction sites of NotI for the Ni and Ci primers, BamHI for the No and EcoRI for the Co primers. Except for *aprX* toxin, HindIII for the No primers were used. The Ni and Ci primers also contained regions of complementary sequence to fuse the 500 bp products together to make a 1 kb product, with the NotI site in the middle. This PCR methodology named as "Splicing by Overlap Extension" (SOE) was used (Horton *et al.,* 1990). This final 1 kb (SOE) product was then ligated into pkmobsacB cloning vector, pre-cut with fast digest restriction enzymes used for knock out (Thermo Fisher Scientific, Scotland, U.K.) according to manufacturer's instructions. Ligations were set up in a 5:1 vector to insert ratio using T4 DNA ligase protocol (Thermo Fisher Scientific, Scotland, U.K.). The pkmobsacB::1kb linker construct was transformed into electro competent DH5α (Invitrogen, Life Technologies, Carlsbad, USA) and plated on LB plates containing selection marker kanamycin, IPTG & X-gal for blue-white screening. Transformed white colonies were recovered at 37 $^{\circ}$C and plasmid isolation done for further assay. To confirm correct size of insert, restriction digestion was performed with appropriate enzymes. This plasmid pK18mobsacB-SOE does not replicate in *Pseudomonas*, therefore a triparental filter mating was performed as previously described using *E. coli* DH10B (pK18mobsacB-SOE) as the donor strain, *E. coli* HB101 (pRK2013) as the helper strain, and *P. poae* as the recipient strain. Integration of the plasmid pK18mobsacB-SOE into the chromosome of *P. poae* by the first crossover was selected on an LB plate supplemented with 50µg mL^{-1} kanamycin (Figure 2.3-1). The second crossover cells were selected by culture on LB plates containing 10 % (w/v in water) sucrose (Figure 2.3-2a,b).All of the constructed strains were validated by PCR and DNA sequencing. Complemented strains were generated by cloning the full length gene into the cloning vector pBBR1MCS-2 followed by triparental conjugation in *P. poae* and selection on KBM plates supplemented with Kanamycin. Complemented strains were verified via PCR as described for the knock-outs.

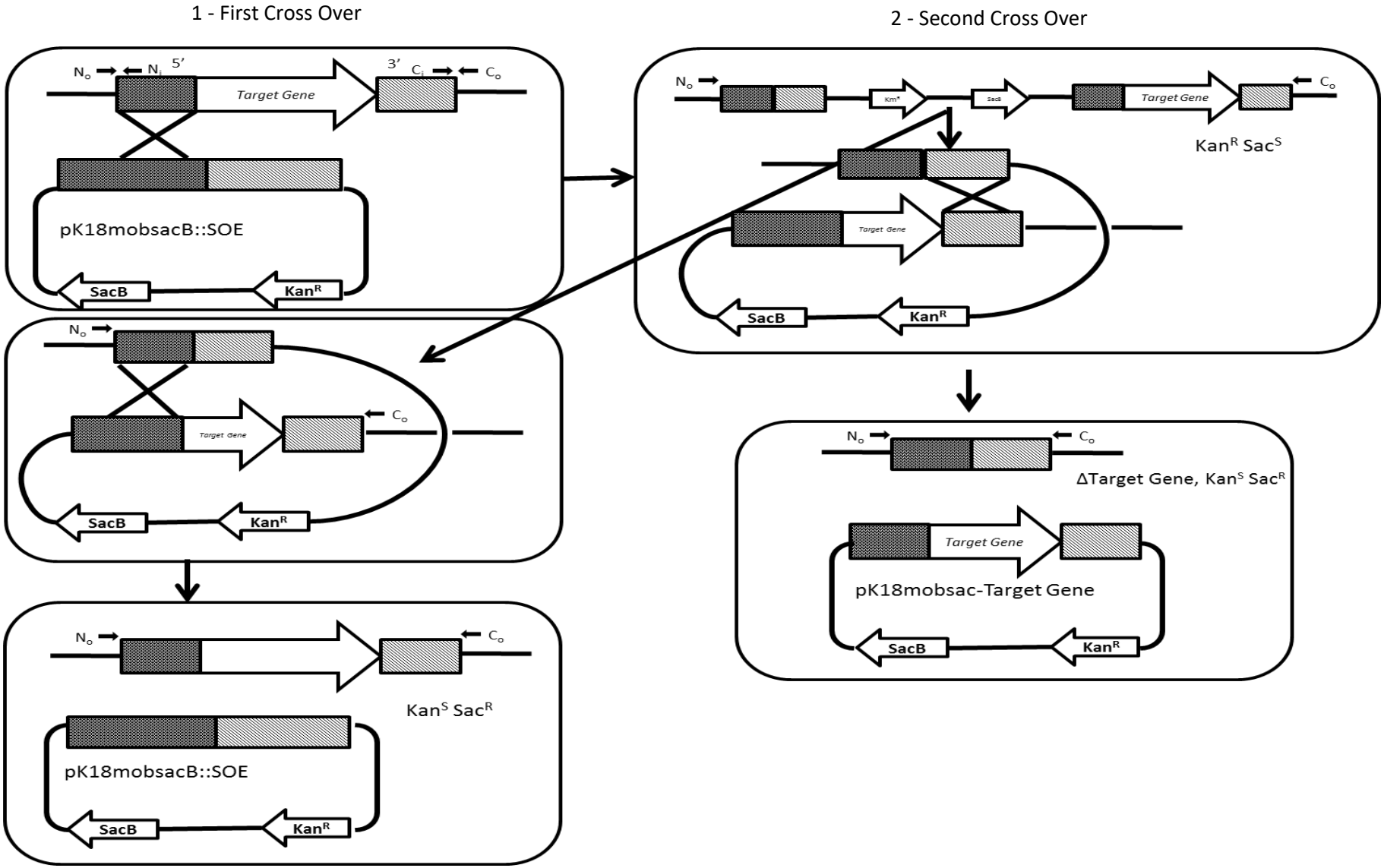


Figure 2.3: Schematic of the gene deletion procedures were conducted in *P. poae*.
1. The "Splicing by Overlap Extension" SOE gene product of selected toxin was constructed in pkmobsacB cloning vector. The first crossover event was occurred in the any of the terminal or 5'/3'arm of target gene in presence of kanamycin marker.

2. Second cross over events occurred in KB + 10 % sucrose medium which yielded two types of strains, Target gene deletion mutants and wild-type revertants.

2a. If second crossover events would take place at other end of target gene than first crossover that resulted in the deletion of gene and finally deleted mutant can recovered from KB plates without any kanamycin and sucrose selection.

2b. In other possibility, when second crossover would occurs at same end of the gene where first crossover happened, which restore the wild type gene.
The validation of deletion mutants was carried out by PCR method through use of No-Co primer pair (deletion primer pairs)

3 Aphid killing bacteria & the susceptibility of different insecticide resistant aphid clones

3.1 Introduction

The peach potato aphid, *Myzus persicae,* is recognized as one of the most important agricultural pests worldwide, in part due to its ability to feed on more than 400 species in 40 different plant families (Blackman & Eastop, 2000; van Emden & Harrington, 2007). *M. persicae* is a major pest on agro-industrial crops (including potato, sugar beet and tobacco), horticultural crops (including plants of Brassicaceae, Solanaceae and Cucurbitaceae families) and stone fruits (peach, apricot, and cherry, among others)(Blackman & Eastop, 2000; Schoonhoven *et al.,*2005). It causes damage to many economically important crop plants through direct feeding, transmitting plant viruses and honey dew production.

The control of *M. persicae* relies almost exclusively on the application of chemical insecticides and their continuous use has resulted in the development of widespread and multiple forms of resistance. In the early 1955's, the first evidence of insecticide resistance was reported in *M. persicae* and over a period of six decades it became strongly resistant to most classes of insecticide, including the organophosphates, carbamates, pyrethroids, cyclodienes, and neonicotinoids (Anthon, 1955; Devonshire, 1998; Bass *et al.,* 2014). Seven distinct mechanisms of resistance have been identified in the green peach aphid:

1. Overproduction of carboxylesterases leading to resistance to organophosphate and carbamate insecticides
2. Mutation of the acetylcholinesterase enzyme conferring insensitivity to dimethyl carbamate insecticides
3. Mutation of the voltage-gated sodium channel resulting in resistance to pyrethroid insecticides
4. Duplication and mutation of the GABA receptor subunit gene conferring resistance to cyclodiene insecticides
5. Overexpression of the cytochrome P450 CYP6CY3 leading to resistance to nicotine and neonicotinoid insecticides
6. Reduced penetration of insecticide through the cuticle associated with resistance to neonicotinoid insecticides
7. Mutation of the nicotinic acetylcholine receptor (nAChR) conferring resistance to neonicotinoid insecticides

Additionally, the inducible mechanism of resistance to insecticide has also been described in *M. persicae.* The study demonstrated that application of a sub-lethal dose of pirimicarb on lab

susceptible and other Kdr & MACE mutant resistant clones resulted in expression of general stress response genes in a susceptible clone and few significant changes in gene expression of resistant mutants (Silva *et al.,* 2012). The reduced stress response of resistant clones implied that they experienced less stress as a consequence of being resistant. Besides these, other studies have described the behaviour modifying effects of insecticides on aphids (Nauen, 1995; Nauen & Elbert, 1997). The recent comparative dispersal assay on neonicotinoid resistant FRC and 5191A clones revealed FRC spent less time on neonicotinoid treated plants compared to untreated plants (Fray *et al.*, 2014).

Although it is clearly understood that resistance to insecticides, either through increased production of metabolic enzymes or alteration of receptors in the insect nervous system, provides clear a benefit to pests under selection with insecticide, it may be associated with fitness costs in the absence of insecticide. This phenomenon is commonly exploited in resistance management and is based on the assumption that, in the absence of insecticides, resistant insects are less 'fit' than their susceptible counterparts due to impairment in the normal functioning of receptor to carry out its native function . Early studies of *M. persicae* provided evidence for some resistant clones moving less readily between different host species in the laboratory (Eggers-Schumacher, 1983). In later studies, the emergence of fitness deficits under field conditions was reported from esterase-overproducing resistant aphids which showed a reduced ability to survive winter conditions (frost, rain and wind) associated with reduced mobility at low temperatures, compared with that of susceptible aphids over winter (Foster *et al.,* 1996). The modified behavioural response of resistant esterase producing aphids are linked to the slow movement of resistant aphids from senescing leaves compared with susceptible aphids (Foster *et al.,* 1997). Another alteration in behavioural response was found in *M. persicae* that carries the Kdr mutation and enhanced esterase production shows a reduced response to aphids' alarm pheromone; furthermore aphids with MACE resistance show a lower reproductive performance compared with susceptible individuals (Foster *et al.,* 1999; Foster, Young, *et al.,* 2003). Hence, a fitness cost is defined as an outcome of trade-offs in energy between traits underlying insecticide resistance and fitness-related traits such as reproduction, development time and adult body size. However, the study on *M. persicae* from Chile reported higher levels of total esterase activity in genotypes carrying at least one Insecticide resistance mechanism (IRM) compared to genotypes without an IRM. This indicated that there is no evidence for energy or reproductive fitness costs associated with total esterase activity or MACE (Castañeda *et al.,* 2011). This research supported a non-random association between insecticide resistance mechanisms rather than tight chromosomal linkage of the resistance genes and contrasts to those of studies that report fitness costs associated with insecticide resistance in *M. persicae*. Moreover, the case of carboxylesterase resistance fitness costs was linked with excessive resource utilization through over-production of

the carboxylesterase enzyme, which accounted for about 3 % of total body protein in very resistant (R3) forms of aphids (Devonshire & Moores, 1982).

The studies described above shows that most insecticides can be compromised by known metabolic and/or target-site resistance mechanisms in this economically important aphid species and highlighting the importance of finding alternative cultural and natural control methods. In regards to the latter it will be important to identify novel biopesticides. The research work showed that bacteria recovered from disease suppressing soils and plant surfaces (phylloplane and rhizosphere) can kill insects. Of these, ten bacterial strains were particularly effective in killing *M*. *persicae* and other aphid species (Livermore, 2016).

My project aim was to characterise these bacterial isolates further, identify the bacterial mechanisms underlying toxicity and the specificity of these bacteria to aphids. The initial screening of bacterial pathogens on different aphid species results in low, moderate or high aphid mortality therefore, it is possible that insecticide-resistant aphids show variations in susceptibility to the bacterial pathogens that might give a clue to the mechanisms of toxicity. Moreover, so far there is no report in literature which states any relationship between fitness of aphid insecticide resistance clone and bacteria challenge.

Therefore it is worth exploring insecticide resistance aphid fitness (more or less) against bacterial challenge. In order to determine this fitness, a collection of *M. persicae* clones with different insecticide resistance mechanisms should be screened for their susceptibility to bacterial challenge compared to insecticide susceptible aphid clones (Table 2.4).

These insecticide resistant aphid clones have enhanced expression of detoxification enzymes due to mutations in insecticide target proteins. Also, some of these aphid mechanisms (may be metabolically costly) confer a fitness penalty, so it may be that the aphids are more sensitive to bacterial infection. However, insect detoxification enzymes have been shown to have a wide array of different xenobiotic substrates. It is hypothesized that resistant clones could be more fit to bacteria challenge due to suppression of bacteria virulence factors by overproduction of detoxification enzymes. The latter is unlikely but possible as important detoxification enzymes have been shown to have a wide array of different xenobiotic substrates.

To examine variations in baseline susceptibility in aphids collected from different locations in Europe, quantal response bioassays were employed to identify mortality response (proportions) at different bacterial doses. This information is useful for statistical comparisons of entire regression lines and individual dose levels of interest.

The main objectives of this chapter were:

1. To identify bacterial isolates that have the potential to cause pathogenicity in aphids using a mortality assay.
2. To observe and compare the susceptibility pattern between “insecticide resistant clones” and “insecticide susceptible clones” against bacterial exposure – Calculating the LC_{50} dose of each bacterium for each aphid clone.
3. To determine if resistant aphids are more or less fit to bacterial challenge by evaluating the resistance ratio of each bacterial strain.
4. To monitor fecundity of different insecticide resistant clones and susceptible clones after bacterial challenge and identify potential differences in reproduction.

3.2 Results

3.2.1 Identification of pathogenic bacterial strains

The Livermore research work showed ten bacterial species were pathogenic to six aphid species at different rates. I aimed to screen a number of different origin aphid genotypes with or without insecticide resistance mechanisms to further evaluate killing efficacy rates of these bacterial pathogens. This initial screening would help in identification of most pathogenic bacteria and also evaluate the susceptibility of various insecticide resistant clones to bacterial challenge. Therefore, an aphid mortality assay with infection dose 10^7 CFU mL^{-1} including insecticide resistant and susceptible aphid clones was carried out. Aphid clones "New green – RES 1", "794J2 – RES 2", "5191A – RES 3" and "5444B – RES 4" along with three susceptible clones "4106A-SUS 1", "4225B-SUS 2" & "Clone-NS SUS-3" were included in the experiment. The results found that six bacterial strains could be classified as 50 % - 100 % pathogenic to all aphid clones while the other four bacterial strains were categorized as "low" and "non-toxic" to all tested aphid clones (Figure 3.1& Table3.1). These six highly virulent aphid killing bacteria were selected for subsequent analysis.

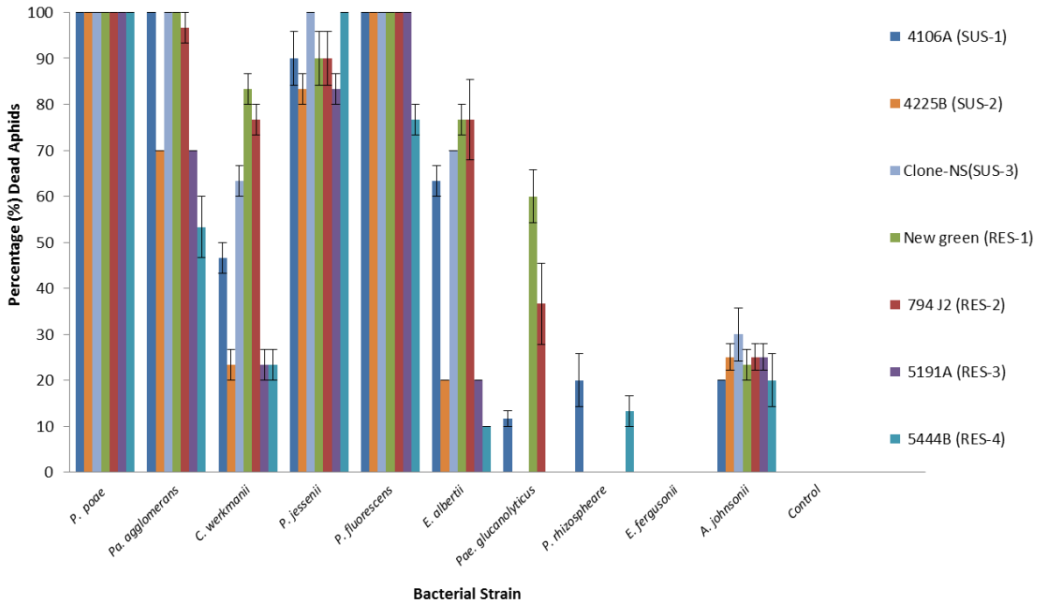


Figure 3.1: Assessment of aphid mortality by various bacterial species. Mortality assay showing the percentage of dead aphids (N=10) at 72 h after ingestion of artificial diet inoculated with various bacterial cells (10^7 CFU mL^{-1}). **Control**: Ten aphids were fed in sterile diet with three replicates. Error bars represent standard error of the mean of three biological replicates. {Aphid clones – three susceptible clones ("4106A-SUS 1", "4225B-SUS 2" & "Clone-NS SUS-3" and four resistant clones "New green – RES 1", "794J2 – RES 2", "5191A – RES 3" and "5444B – RES 4"} Note: "5191A – RES 3" and "5444B – RES 4" were not tested with *Paenibacillus glucanolyticus*

Table 3.1: Summary of bacterial toxicity on the aphid clones.							
Bacterial Strains	**4106A (Susceptible clone)**	**4225B (Susceptible clone-2)**	**Clone-NS (Susceptible clone-3)**	**794J2 (UK Resistance clone)**	**New green (UK Resistance clone)**	**5191A (Resistance clone)**	**5444B (Resistance clone)**
P. poae	++++	++++	++++	++++	++++	++++	++++
Pa. agglomerans	++++	++++	++++	++++	++++	+++	++
P. fluorescens	++++	++++	++++	++++	++++	+++	+++
P. jessenii	++++	++++	++++	++++	++++	++++	++++
E. albertii	++++	++	+++	++++	++++	+	+
C. werkmanii	+++	++	+++	+++	+++	+	+
A. johnsonii	+	---	++	+	+	+	+
P. rhizosphaerae	+	---	---	---	---	---	+
Pae. glucanolyticus	---	---	---	++	++	Not tested	Not tested
E. fergusonii	---	---	---	---	---	---	---
Seven aphid clones after ingestion of artificial diet inoculated with bacteria dose 10^7 CFU mL^{-1} at 72 h showed high toxicity **(++++)** i.e. 90-100 % death, moderate toxicity **(+++)** i.e. 50-80 % death, low toxicity **(++)** i.e. 30-50% death, lowest toxicity **(+)** i.e. 10-30 % death and no toxicity or were non-pathogenic. **(---) Not tested**.							

3.2.2 Effect of bacterial cells concentrations on the mortality of different insecticide aphid clones

An important consideration is to determine the relative toxicity of each bacterial strain as this can help decide the best choice of bacterium to use in biocontrol treatments. Thus, for three days, three biological replicates of all bacteria were tested at six concentrations ranging from 10^7 CFU mL^{-1} to 10^2 CFU mL^{-1} to evaluate the mortality patterns in the different insecticide clones. Evaluation of bacterial susceptibility in various aphid clones (insecticide susceptible and resistant) at different time points was carried out. Such rigorous assessment enabled us to determine which aphid clones were more or less fit to bacteria challenge. The main observations of aphid mortality at various cell concentrations were further examined at 48 and 72 h which help in assessing variance of bacterial toxicity.

3.2.2.1 Assessment of aphid mortality at 48 h

The initial 72 h screening results revealed variation in the mortality rate of different aphid clones to the six most pathogenic bacteria at 10^7 CFU mL^{-1}. The next step was to examine aphid mortality patterns with the same infection dose of the six bacterial strains at 48 h.

Pseudomonas poae and Pseudomonas fluorescens produced the highest mortality (90-100 %) in all the UK resistant and susceptible clones after 48 h. In the case of *Pantoea agglomerans* 20-80 % mortality was observed in all the UK resistant and susceptible clones. Three other bacterial species *Pseudomonas jessenii, Citrobacter werkmanii* and *Escherichia albertii* produced 20-40 % death only in the UK resistant and susceptible clones (Figure 3.2, 3.3 A-E). At higher infection dose (10^7 CFU mL^{-1}), two clones from mainland Europe**,** 5191A and 5444B, were both less sensitive to all *Pseudomonas* strains with 20-70 % mortality (Figure 3.3 F & G).

For all bacteria 10^5-10^6 CFU mL^{-1} produced 20-100 % death in all UK insecticide resistant aphid clones and below that concentration no mortality was observed (Figure 3.3 A-E). Similarly, no death was observed in 5191A and 5444B at 10^5-10^6 CFU mL^{-1} doses for all bacterial strains. No aphid mortality was recorded in any control sachets (Mittler diet without bacteria). The mean values of all different aphid mortality were tested by two way ANOVA followed by comparison of means by Tukey-Kramer HSD test, using GenStat software.

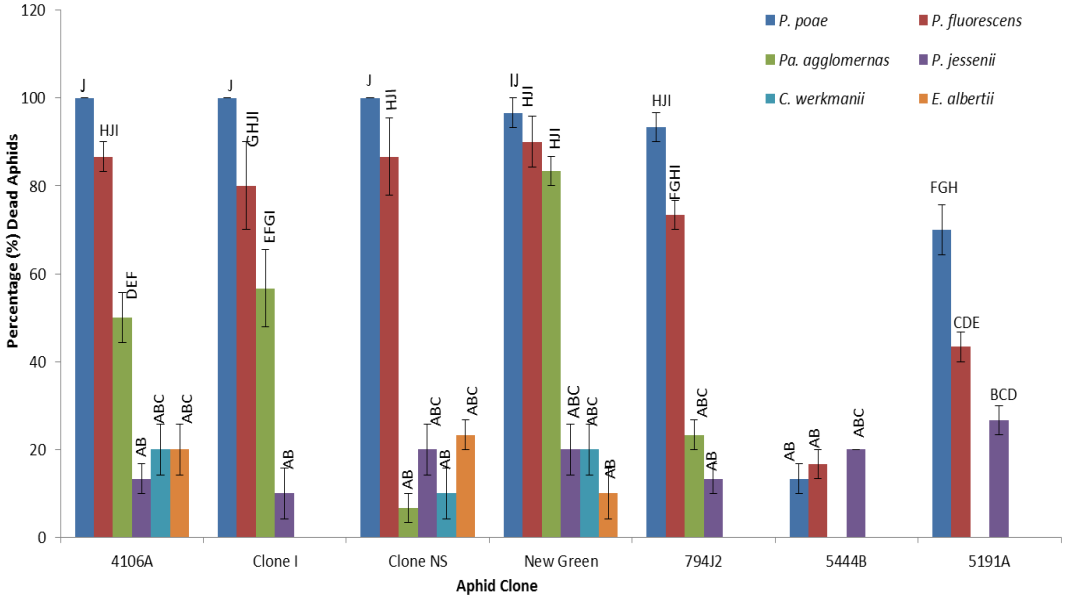


Figure 3.2: Assessment of aphid mortality by various bacterial cells. Mortality assay showing the percentage of dead aphids (N=10) at 48 h after ingestion of artificial diet inoculated with cells of various bacterial species (10^7 CFU mL^{-1}). Error bars represent standard error of the mean of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD were shown as letters (where different letters on the graphs indicate statistically significant differences).

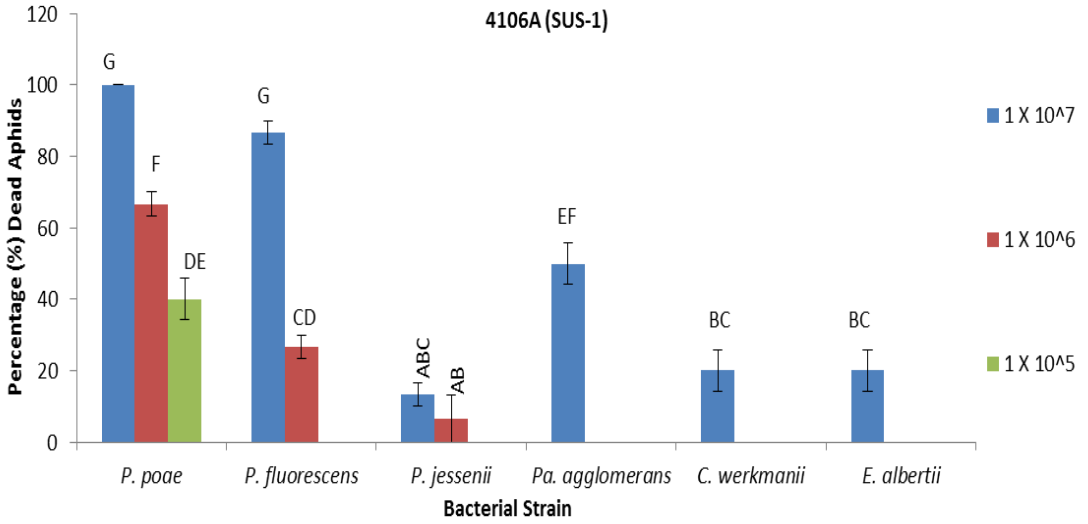


Figure 3.3 A: Effect of bacterial concentration on aphid mortality for 4106A (SUS-1) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

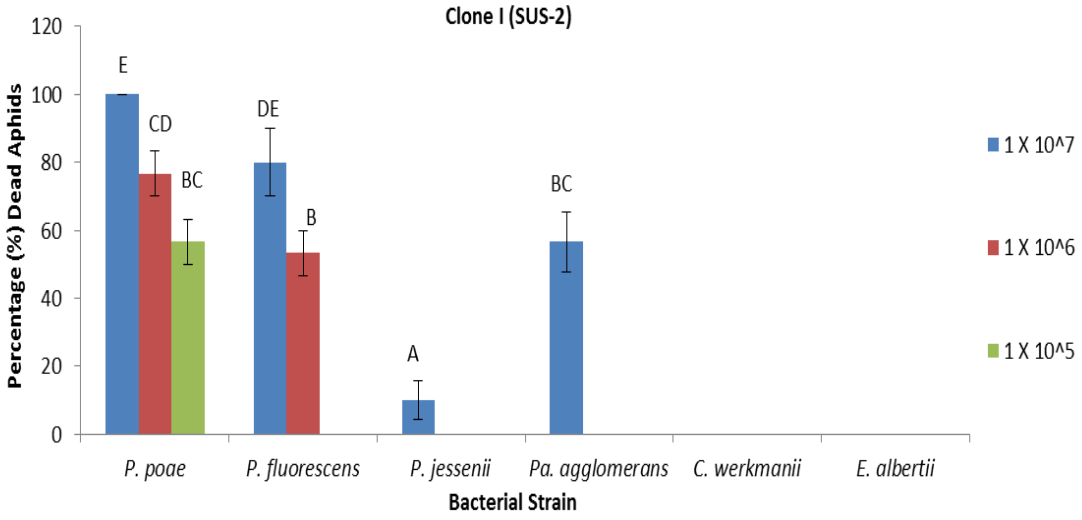


Figure 3.3 B: Effect of bacterial concentration on aphid mortality for Clone I (SUS-2) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

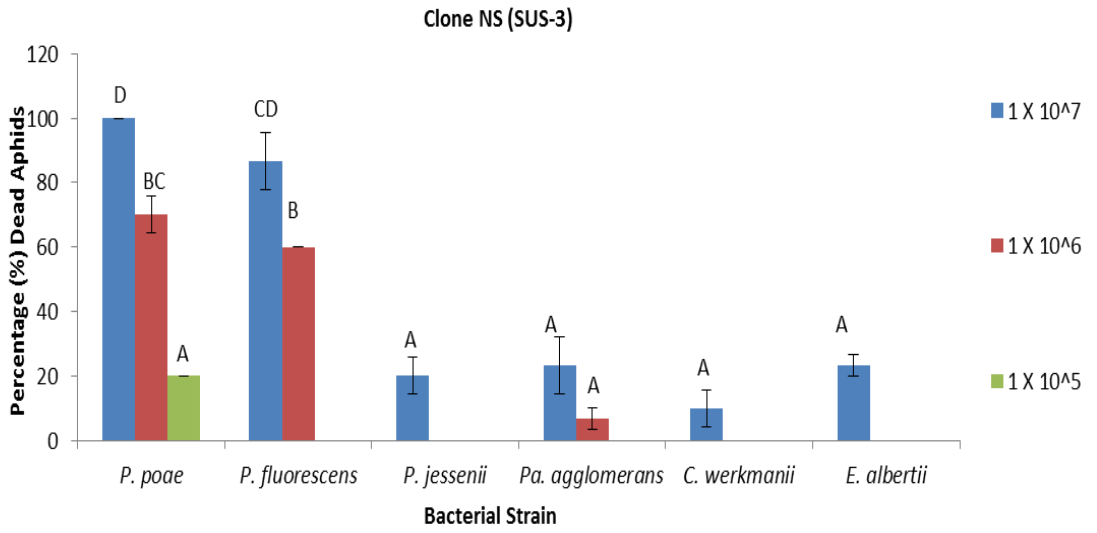


Figure 3.3 C: Effect of bacterial concentration on aphid mortality for Clone NS (SUS-3) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

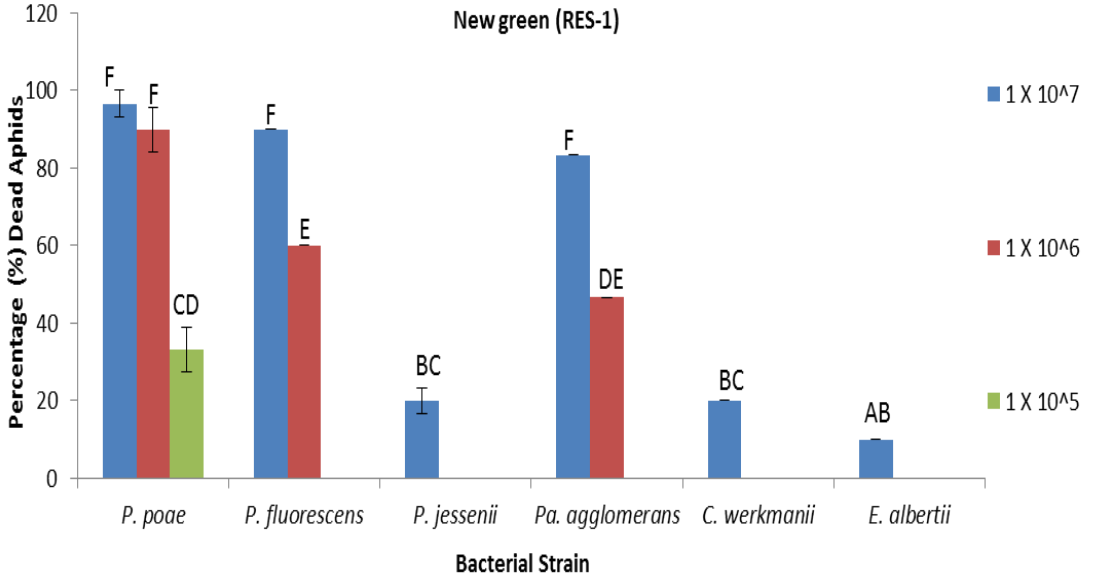


Figure 3.3 D: Effect of bacterial concentration on aphid mortality for New green (RES-1) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

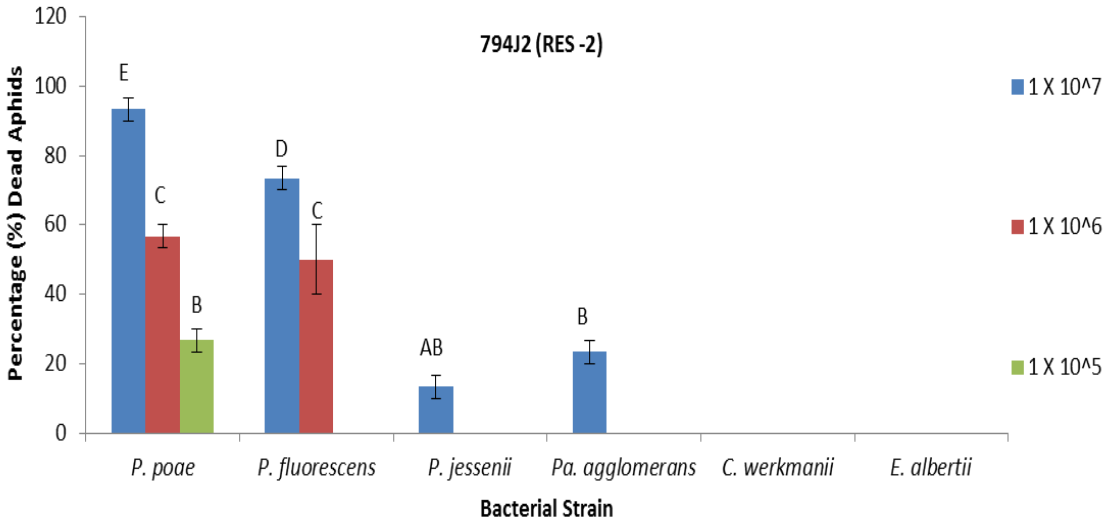


Figure 3.3 E: Effect of bacterial concentration on aphid mortality for 794J2 (RES-2) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences

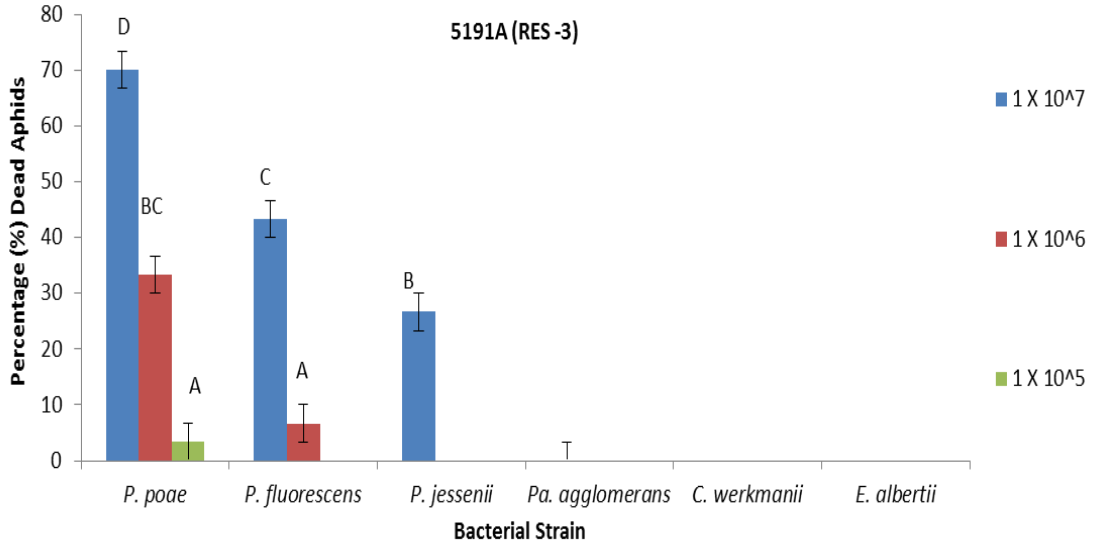


Figure 3.3 F: Effect of bacterial concentration on aphid mortality for 5191A (RES-3) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

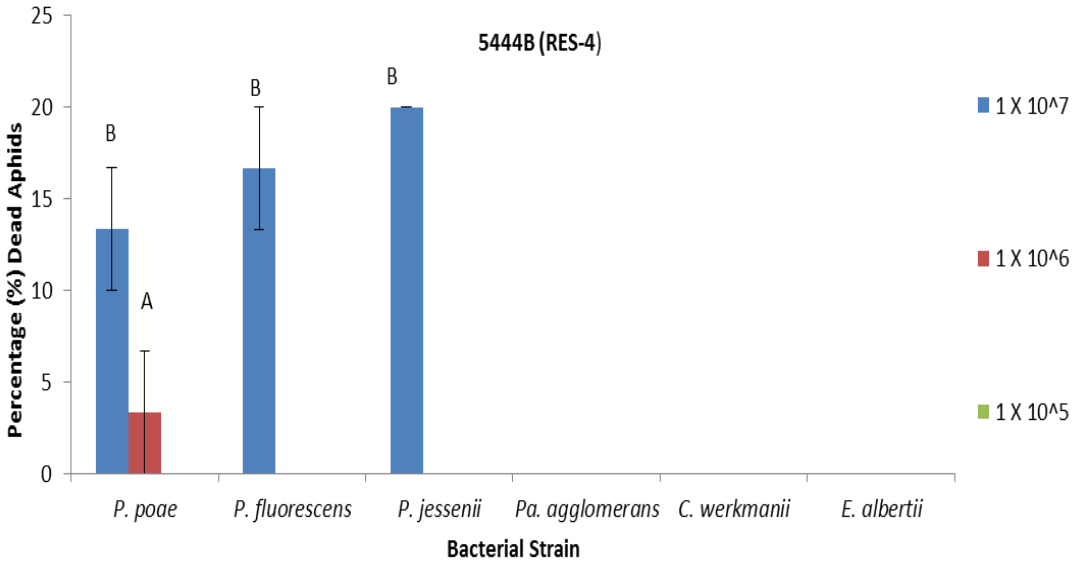


Figure 3.3 G: Effect of bacterial concentration on aphid mortality for 5444B (RES-4) aphid clone after 48 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} (blue bars), for 48 h. No death was reported in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

3.2.2.2 Assessment of aphid mortality at 72 h

After 72 h, all six strains *Pseudomonas poae*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, and *Pseudomonas jessenii*, *Citrobacter werkmanii* and *Escherichia albertii* caused 80-100 % aphid mortality. They were toxic to all three sensitive aphid clones (4106A, 4225B & clone-NS) and the other two UK insecticide resistant aphid clones at bacterial cell concentrations ranging from 10^5 to 10^7 CFU mL^{-1}; at lower concentrations they showed 20-50 % mortality rate against all aphid clones (Figure 3.4 A-E). In addition to this, *Citrobacter werkmanii* and *Escherichia albertii* were responsible for 60-80 % death in all UK and sensitive clones at 10^7 CFU mL^{-1}; at lower concentrations they caused 20-50 % mortality rate (Figure 3.4 A-E).

In the case of 5191A (RES - 3) and 5444B (RES-4), only three strains, *Pseudomonas poae*, *Pseudomonas fluorescens* and *Pseudomonas jessenii,* caused 40-100 % mortality at 10^6 to 10^7 CFUmL^{-1} and lower concentrations caused only 20-30 % mortality (Fig. 3.4 F& G). *Pantoea agglomerans* was considered as moderately pathogenic to 5191A (RES- 3) and 5444B (RES-4) and caused 70 % and 50 % mortality, respectively. Other strains like *Citrobacter werkmanii* and

Escherichia albertii were identified as less efficacious against these clones and caused only 10-20 % mortality at 10^7 CFU mL^{-1} (Fig. 3.4 F& G).

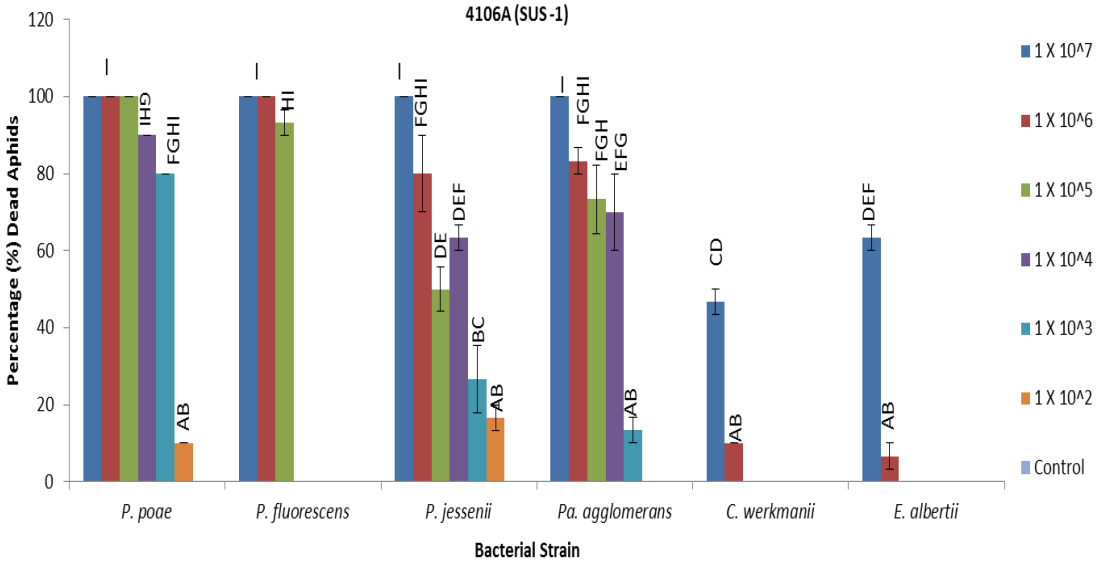


Figure 3.4 A: Effect of bacterial concentration on aphid mortality for 4106A (SUS-1) aphid clone after 72 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

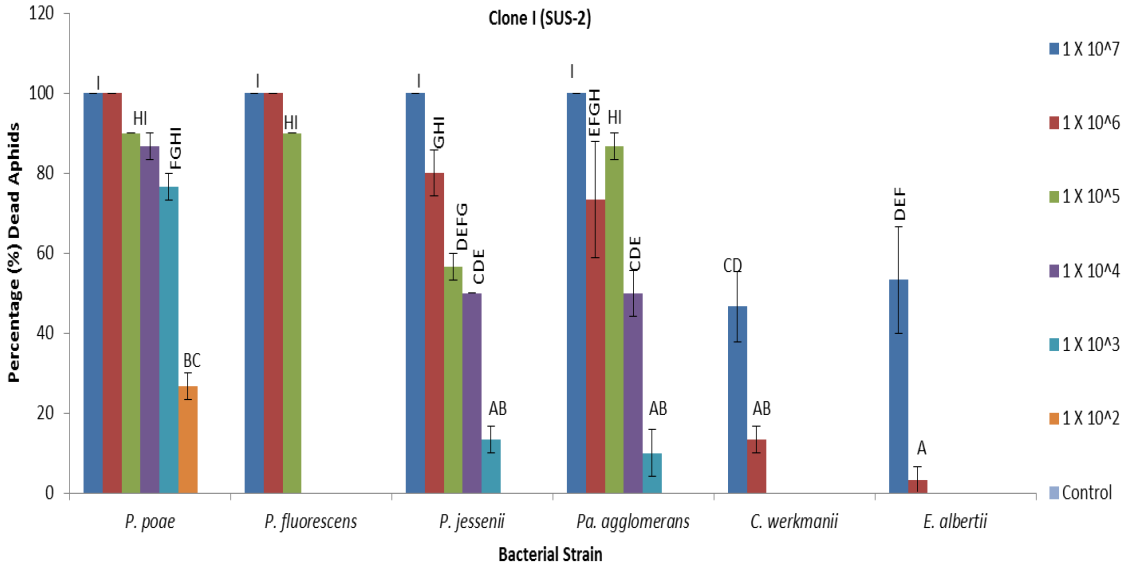


Figure 3.4 B: Effect of bacterial concentration on aphid mortality for Clone I (SUS-2) aphid clone after 72 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

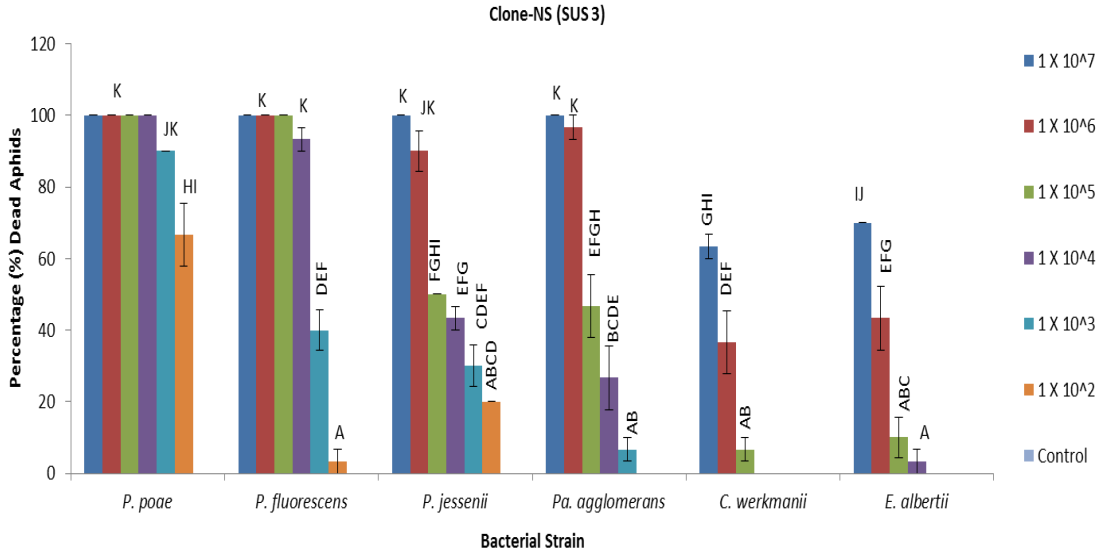


Figure 3.4 C: Effect of bacterial concentration on aphid mortality for Clone NS (SUS-3) aphid clone after 72 h. Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

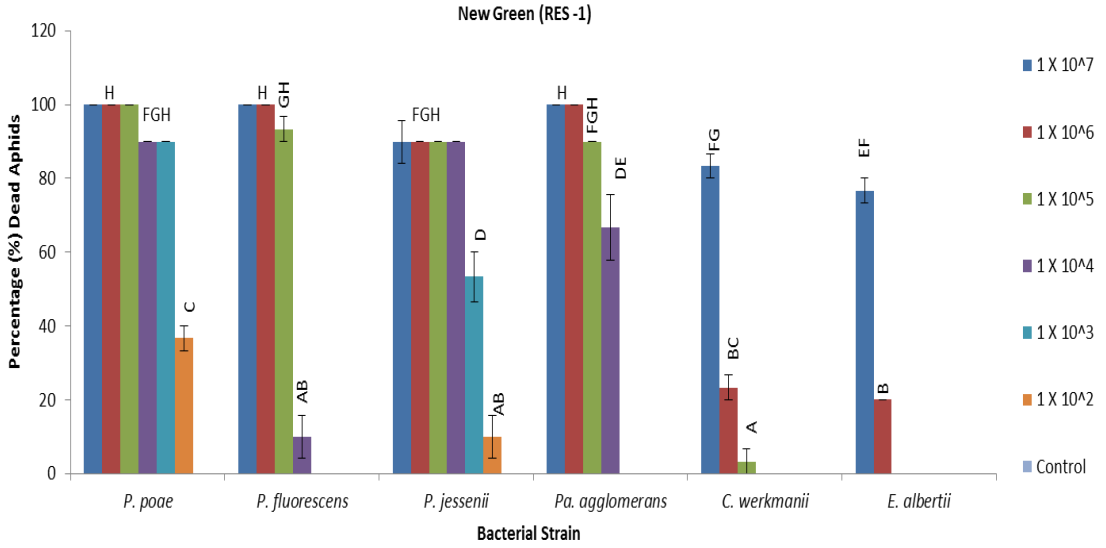


Figure 3.4 D: Effect of bacterial concentration on aphid mortality for New green (RES-1) aphid clone after 72 h. Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

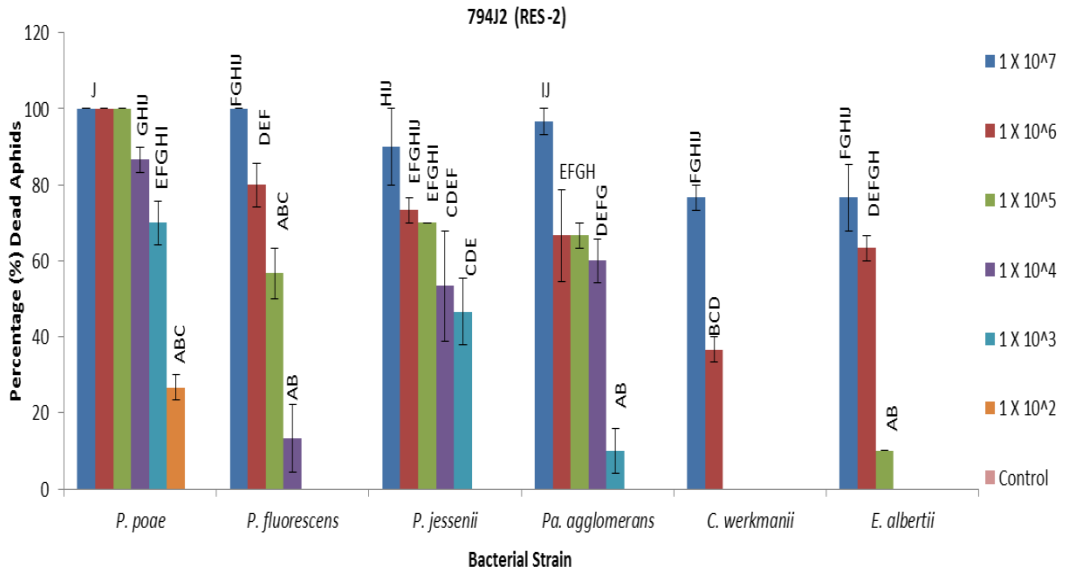


Figure 3.4 E: Effect of bacterial concentration on aphid mortality for 794J2 (RES-2) aphid clone after 72 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

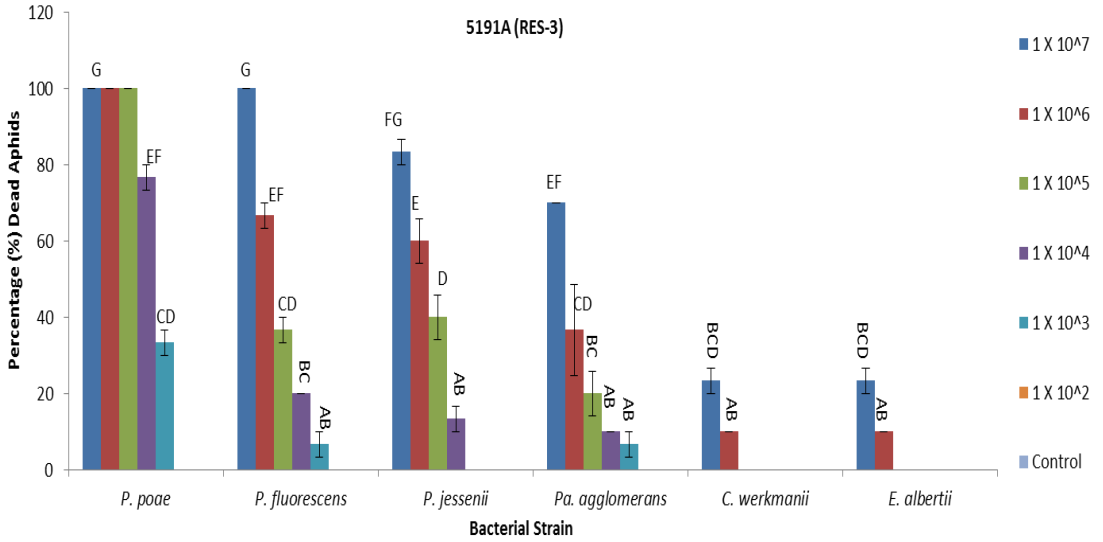


Figure 3.4 F: Effect of bacterial concentration on aphid mortality for 5191A (RES-3) aphid clone after 72 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

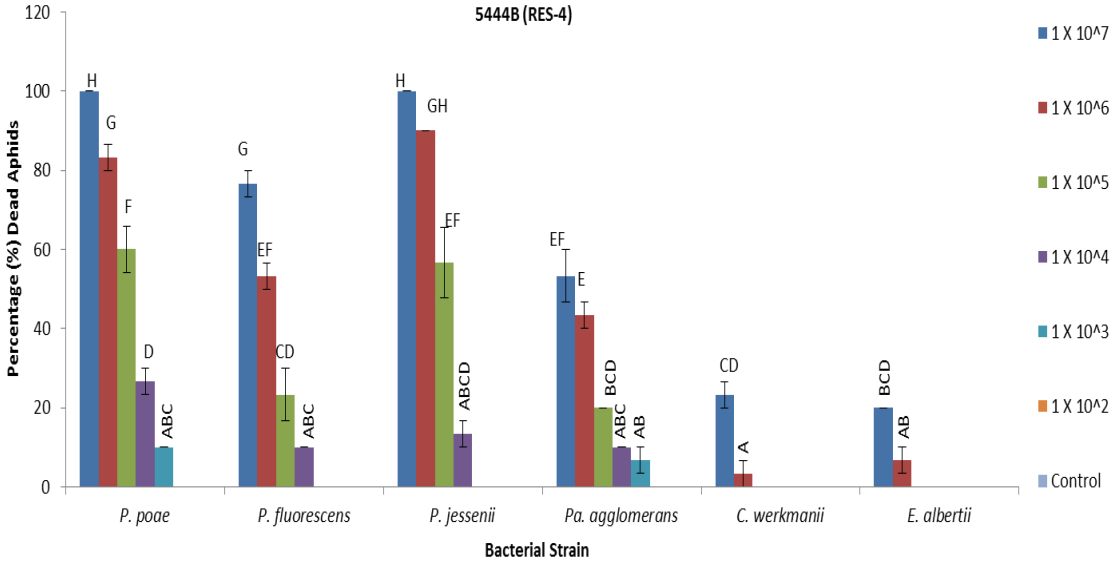


Figure 3.4 G: Effect of bacterial concentration on aphid mortality for 5444B (RES-4) aphid clone after 72 h.
Aphid mortality assay showing the percentage (N = 10) of dead aphids after ingestion of artificial diet inoculated with various bacterial species cells at 1 x 10^2 CFU mL^{-1} (orange bars), 1 x 10^3 CFU mL^{-1} (light blue bars), 1 x 10^4 CFU mL^{-1} (purple bars), 1 x 10^5 CFU mL^{-1} (green bars), or 1 x 10^6 CFU mL^{-1} (red bars), or 1 x 10^7 CFU mL^{-1} dark blue bars), for 72 h. No death was observed in control and lower concentration treated sachets. The data presented are the mean and standard error of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD are shown as letters (different letters on the graphs) indicate statistically significant differences.

3.2.2.3 Statistical inference on aphid mortality assays

To generalise pathogenicity of various bacteria on different insecticide and sensitive aphid clones, a statistical linear model was created using the "Fit-Model" tool in JMP software. In this tool, a model was generated between the mean values of mortality readings (72 h) of all aphid clones for each bacterium treatment with other parameters like bacteria strains, aphid clones and different doses (Table 3.2). The effect of all parameters on aphid mortality showed variation with large significant difference at p value 0.001. However, the bacteria with dose and aphid clone interactions displayed a significant difference on aphid mortality of different clones (*$p<0.05$). The reason for the large effect on aphid mortality from bacteria – dose parameters were due to a linear relationship between dose and mortality for all bacterial strains.

The aphid clone * bacteria interaction in this model defined a general trend of all bacteria potency to kill different aphid clones from Squares (LS) means of aphid mortality (mean values estimated by linear model) to lower LS mean values (Table 3.3). *P. poae* was classified as the most pathogenic bacterium, which was responsible for mortality in all sensitive and UK insecticide resistant aphid clones with their LS mean values of mortality ranging from 80-90 followed by 68 in

5191A and 46 in the 5444B resistance clone. *P. fluorescens*, *P. jessenii* and *Pa. agglomerans* were categorised as intermediate killing efficacy as their LS mean values ranged from 45 to 70 for most aphid clones with an exception of lower values from 22 to 43 for both European insecticide resistant clones. *Citrobacter werkmanii* and *Escherichia albertii* were identified as less efficacious against these clones with LS mean values from 4 to 21 and they were thus ranked as the least virulent bacteria.

Table 3.2: Summary of Generalized linear model.

Source	Degrees of Freedom (DF)	Sum of Squares	Variance Ratio	Prob > F
Aphid clone	6	66810.05	255.87	<.0001
Bacteria	5	380418.52	1748.31	<.0001
Dose	5	511894.71	2352.54	<.0001
Aphid clone*Bacteria*Dose	150	94921.16	14.54	<.0001
Aphid clone*Bacteria	30	29707.41	22.75	<.0001
Aphid clone*Dose	30	9942.33	7.62	<.0001
Bacteria*Dose	25	108030.69	99.30	<.0001

Table 3.3: Generalized linear model of aphid mortality on exposure of various pathogenic bacteria.																	
Aphid clone, Bacterium	**Letters for Significance**																**Least Sq Mean Values of aphid mortality**
Clone-NS, P. poae	A																92.78
New green, P. poae	A	B															86.11
794J2, P. poae		B	C														80.56
Clone-I, P. poae		B	C	D													80.00
4106A, P. poae		B	C	D													80.00
Clone-NS, P. fluorescens			C	D													72.78
New green, P. jessenii			C	D	E												70.56
5191A, P. poae				D	E	F											68.33
New green, Pa. agglomerans					E	F	G										59.44
4106A, Pa. agglomerans						F	G	H									56.67
4106A, P. jessenii							G	H									56.11
794J2, P. jessenii							G	H									55.56
Clone-NS, P. jessenii							G	H									55.56
Clone-I, Pa. agglomerans							G	H	I								53.33
New green, P. fluorescens							G	H	I								50.56
Clone-I, P. jessenii							G	H	I	J							50.00
794J2, Pa. agglomerans							G	H	I	J							50.00
4106A, P. fluorescens							G	H	I	J							48.89
Clone-I, P. fluorescens							G	H	I	J							48.33
5444B, P poae								H	I	J							46.67
Clone-NS, Pa. agglomerans								H	I	J							46.11
5444B, P. jessenii									I	J	K						43.33
794J2, P. fluorescens									I	J	K						41.67
5191A, P. fluorescens										J	K	L					38.33
5191A, P. jessenii											K	L	M				32.78
5444B, P. fluorescens												L	M	N			27.22
794J2, E. albertii													M	N			25.00
5191A, Pa. agglomerans													M	N			23.89
5444B, Pa. agglomerans													M	N	O		22.22
Clone-NS, E. albertii													M	N	O	P	21.11
794J2, C. werkmanii														N	O	P	18.89
New green, C. werkmanii														N	O	P	18.33
Clone-NS, C. werkmanii														N	O	P	17.78
New green, E. albertii														N	O	P	Q 16.11
4106A, E. albertii															O	P	Q 11.67
Clone-I, C. werkmanii																P	Q 10.00
4106A, C. werkmanii																P	Q 9.44
Clone-I, E. albertii																P	Q 9.44
5191A, C. werkmanii																	Q 5.56
5191A, E. albertii																	Q 5.56
5444B, C. werkmanii																	Q 4.44
5444B, E. albertii																	Q 4.44

The table represents least squares means values of aphid mortality after 72 h exposure of various bacteria treatment at different levels analysed by Student t-test. The least squares means values of aphid clone-bacterium not connected by same letters were statistically significant (*p<0.05) to each other. {Least square means defined as linear combination (sum) of the estimated effects (means) from a linear model}

3.2.3 Determination of LC_{50} (lethal concentration 50) values for all aphid clones

The next step was to evaluate the susceptibility patterns of insecticide resistant and susceptible aphid clones and investigate whether resistant clones are more or less fit towards bacterial challenge. Therefore, a Lethal Concentration – 50 (LC_{50} – the concentration which kills 50 % of the test population) was calculated for each aphid clone to compare the susceptibility of different clones and estimate a 'Resistance ratio' which is a ratio of the LC_{50} value of the resistant clone with the LC_{50} value of the susceptible clone. To calculate LC_{50} values of each bacterium for all aphid clones, 72 h aphid mortality readings at six bacterial concentrations ranging from 10^7 CFU mL^{-1} to 10^2 CFU mL^{-1} were transformed to mortality probits through the use of the (GenStat 15th edition) probit analysis tool, which produced a line of regression. This linear relationship between mortality probits and log of concentration was further imported in GenStat to determine effective LC_{50} doses of all aphid clones for each bacterium. The figure (Appendix Figures 1 to 7) showed an example of linear relationship between each aphid clone mortality probits versus various bacterial doses. Similarly, mortality probits and LC_{50} values were calculated for all aphid clones (42 in total) of their individual bacteria treatment.

At 72 h of bacterial exposure, the LC_{50} value of the individual aphid clone (Figure 3.5, 3.6, 3.7 & 3.8) provides potential variations in their bacterial susceptibility which enables an estimate of the resistance factor of different aphid clones.

The standardization of the aphid mortality assay was performed on the standard UK origin susceptible clone 4106, which generated a consistent and reliable dataset. Therefore, to examine variations in baseline susceptibility in resistant aphid clones, the UK origin susceptible clone 4106 was considered as the reference aphid clone for calculating the resistance ratio.

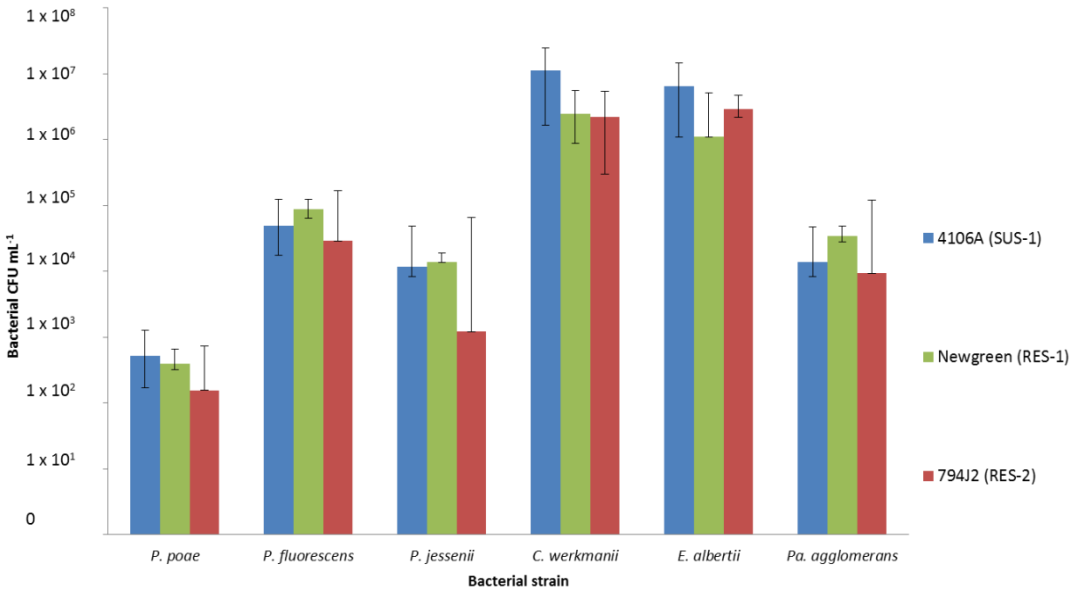


Figure 3.5: Susceptibility of UK insecticide resistant aphid clones to bacterial challenge compared to a reference 4106A aphid susceptible clone. LC_{50} values for different bacteria (N = 3 biological replicates) are shown for each of the three aphid clones. The results showed no statistical significance ($p<0.05$) in LC_{50} values due to overlapping upper and lower doses for each of the clones in all bacterial treatments. The error bar represents the lower and upper values of LC_{50} dose at 95 % confidence limits.

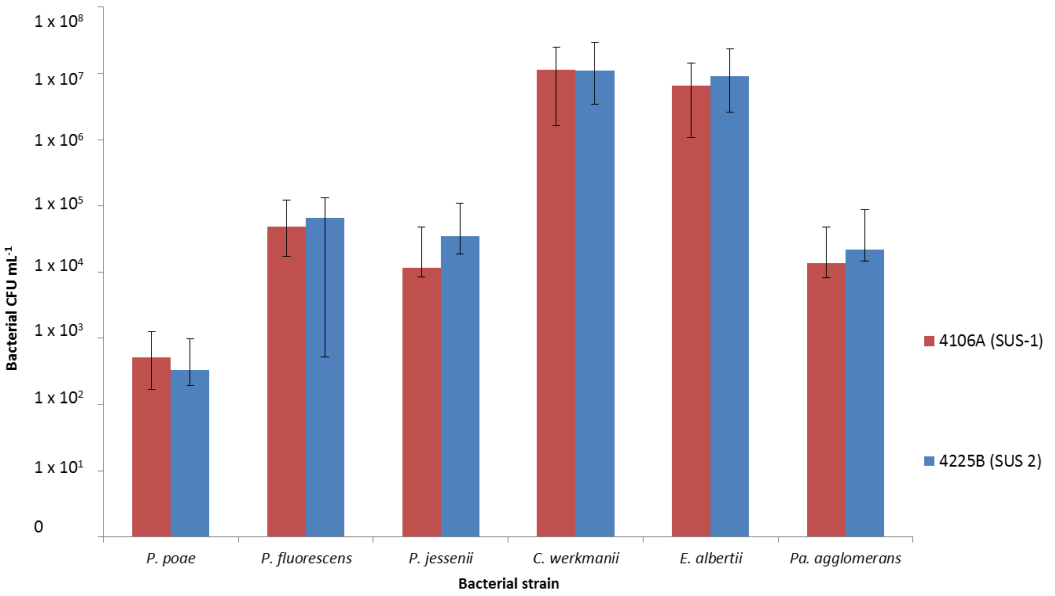


Figure 3.6: Susceptibility of UK susceptible aphid clone to bacterial challenge compared to a reference 4106A aphid susceptible clone. LC_{50} values for different bacteria (N = 3 biological replicates) are shown for each of the two aphid clones. The results showed no statistical significance ($p<0.05$) in LC_{50} values due to overlapping upper and lower doses for each of the clones in all bacterial treatments. The error bar represents the lower and upper values of LC_{50} dose at 95 % confidence limits.

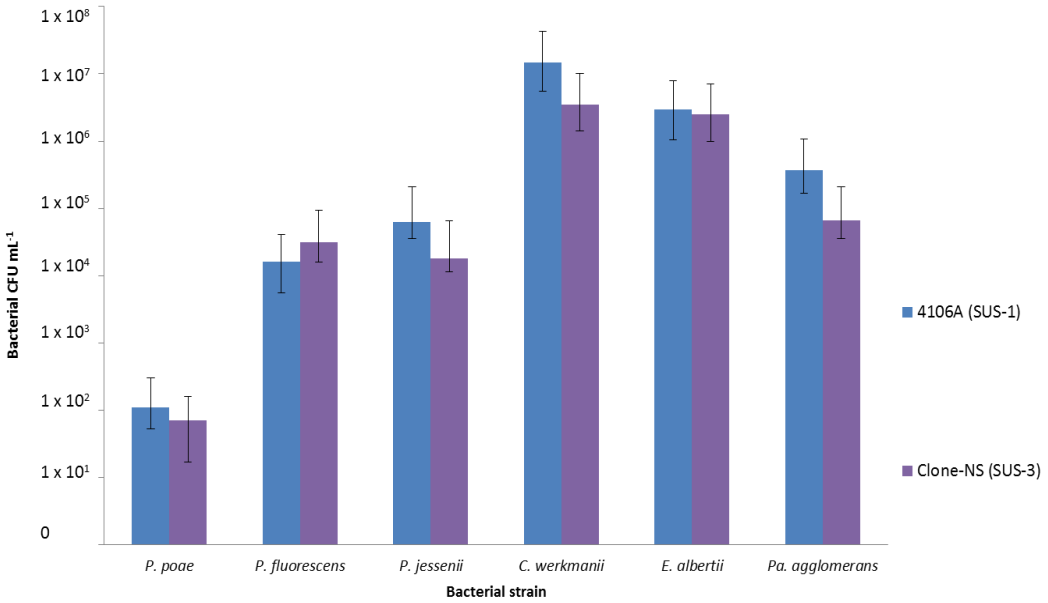


Figure 3.7: Susceptibility of Europe susceptible clone-NS to bacterial challenge compared to a reference 4106A aphid susceptible clone. LC_{50} values for different bacteria (N = 3 biological replicates) are shown for each of the two aphid clones. The results showed no statistical significance ($p<0.05$) in LC_{50} values due to overlapping upper and lower doses for each of the clones in all bacterial treatments. The error bar represents the lower and upper values of LC_{50} dose at 95 % confidence limits

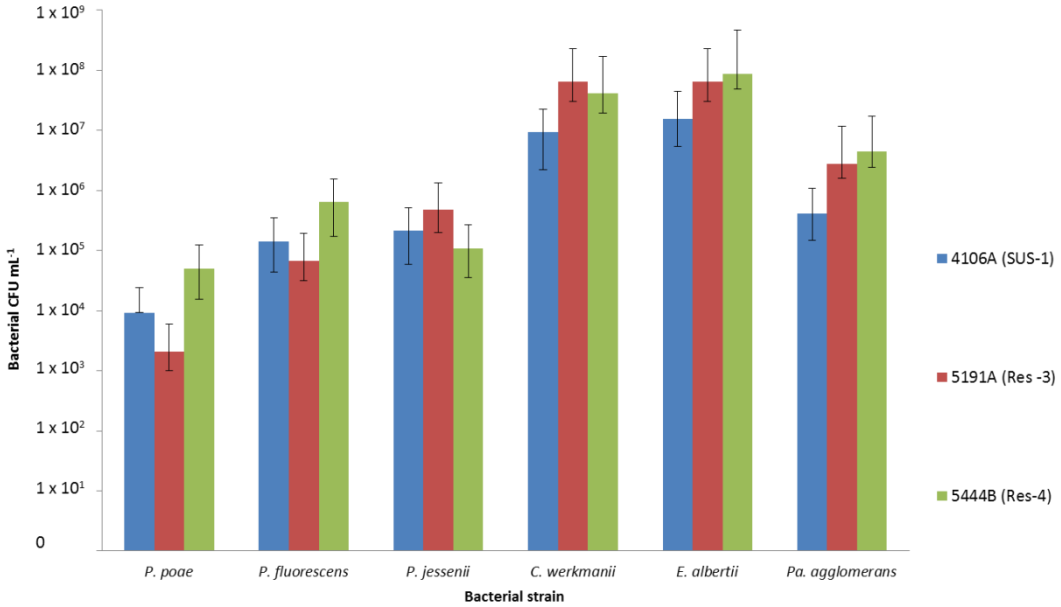


Figure 3.8: Susceptibility of Europe mainland insecticide resistant aphid clones to bacterial challenge compared reference 4106A aphid susceptible clone. LC_{50} values for different bacteria (N = 3 biological replicates) are shown for each of the three aphid clones. The results showed no statistical significance ($p<0.05$) in LC_{50} values due to overlapping upper and lower doses for each of the clones in all bacterial treatments. The error bar represents the lower and upper values of LC_{50} dose at 95 % confidence limits.

The resistance factor of the New green (RES 1) aphid for all six pathogenic bacteria was lower than 1.00 (Table 3.4), which indicates it has a greater susceptibility to bacterial challenge than the reference susceptible clone 4106A. However, another UK resistant clone 794J2 (RES 2) showed variance in susceptibility towards different bacteria. 794J2 (RES 2) had a lower resistance ratio

(less than 1) for *P. poae*, *C. werkmanii* and *E. albertii* whereas it was slightly resistant (1.8-2.5 fold) to P. *fluorescens* and *Pa. agglomerans* compared to reference susceptible clone 4106A.

The aphid clone 5444B was the most resistant to all bacterial species except for *Pseudomonas jessenii* where it was more sensitive than 4106A with a RF of 0.49. Another European aphid clone 5191A was also more sensitive to both *Pseudomonas poae* and *Pseudomonas fluorescens* than the reference susceptible clone 4106A with resistance factor of 0.22 and 0.47, respectively. For the other four bacterial species this clone was identified as more resistant with a greater resistance factor (Table 3.5).

To strengthen any correlation between bacterial and insecticidal susceptibility two more susceptible reference clones (4225B and Clone-NS) were tested. The UK derived 4225B clone showed greater susceptibility (RF=0.64) than 4106A to *P. poae*. 4225B was found to be more resistant to *P. jessenii* challenge than 4106A with a resistance factor of 3. However, similar resistance ratios for the rest of the bacteria *as* compared to reference 4106A clone were observed (Table 3.6).

The resistance factor of the European derived Clone-NS for all pathogenic bacteria was lower than 1, which indicated greater susceptibility towards all bacterial challenge than the reference susceptible clone 4106A with an exception of slight resistance to *P. fluorescens* (RF of 1.92) (Table 3.7).

Table 3.4: Bioassay results with different bacteria against UK insecticide susceptible and resistant aphid clones.

Aphid clones	4106A (SUS-1)		New green (RES 1)			794J2 (RES 2)		
Bacteria	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**Resistance Factor***	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**Resistance Factor***
Pseudomonas poae	5.22×10^2	$3.5 \times 10^2 – 7.55 \times 10^2$	1.55×10^2	$7.7 \times 10^1 – 2.62 \times 10^2$	0.30	3.99×10^2	$2.38 \times 10^3 – 5.23 \times 10^3$	0.77
Pseudomonas fluorescens	4.87×10^4	$3.13 \times 10^4 – 7.45 \times 10^4$	2.89×10^4	$2.31 \times 10^4 – 3.62 \times 10^4$	0.59	8.77×10^4	$1.63 \times 10^4 – 4.98 \times 10^4$	1.80
Pseudomonas jessenii	1.16×10^4	$3.22 \times 10^3 – 3.68 \times 10^4$	1.22×10^3	$1.32 \times 10^2 – 5.1 \times 10^3$	0.10	1.37×10^4	$2.47 \times 10^3 – 1.59 \times 10^4$	1.18
Citrobacter werkmanii	1.12×10^7	$9.57 \times 10^6 – 1.34 \times 10^7$	2.2×10^6	$1.59 \times 10^6 – 3.13 \times 10^6$	0.20	2.47×10^6	$1.97 \times 10^4 – 7.28 \times 10^4$	0.22
Escherichia albertii	6.53×10^6	$5.44 \times 10^6 – 7.97 \times 10^6$	2.89×10^6	$2.12 \times 10^6 – 4.07 \times 10^6$	0.44	1.11×10^6	$3.54 \times 10^3 – 2.44 \times 10^4$	0.17
Pantoea agglomerans	1.37×10^4	$5.38 \times 10^3 – 3.375 \times 10^4$	9.4×10^3	$6.19 \times 10^3 – 1.43 \times 10^4$	0.69	3.43×10^4	$3.17 \times 10^3 – 1.57 \times 10^4$	2.51

The LC_{50} dose of each bacterium for each aphid clone is shown along with lower and upper concentrations values at 95 % confidence limits. The calculated resistance factor for the two resistant aphid clones is also shown. *Resistance factor is a ratio of the LC_{50} value of the resistant clone to the LC_{50} value of the susceptible clone.

Table 3.5: Bioassay results with different bacteria against susceptible and Europe insecticide resistant aphid clones.

Aphid clones	4106A (SUS-1)		5191A (RES 3)			5444B (RES 4)		
Bacteria	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**Resistance Factor***	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**Resistance Factor***
Pseudomonas poae	9.28×10^3	$5.77 \times 10^3 – 1.49 \times 10^4$	2.08×10^3	$1.09 \times 10^3 – 3.85 \times 10^3$	0.22	4.95×10^4	$3.39 \times 10^4 – 7.23 \times 10^4$	5.33
Pseudomonas fluorescens	1.42×10^5	$9.81 \times 10^4 – 2.06 \times 10^5$	6.73×10^4	$3.60 \times 10^4 – 1.27 \times 10^5$	0.47	6.51×10^5	$4.81 \times 10^5 – 8.97 \times 10^5$	4.58
Pseudomonas jessenii	2.14×10^5	$1.56 \times 10^5 – 2.96 \times 10^5$	4.81×10^5	$2.82 \times 10^5 – 8.63 \times 10^5$	2.24	1.07×10^5	$7.12 \times 10^4 – 1.60 \times 10^5$	0.50
Citrobacter werkmanii	9.43×10^6	$7.19 \times 10^6 – 1.32 \times 10^7$	6.38×10^7	$3.40 \times 10^7 – 1.62 \times 10^8$	6.77	4.17×10^7	$2.26 \times 10^7 – 1.30 \times 10^8$	4.42
Escherichia albertii	1.57×10^7	$1.04 \times 10^7 – 2.84 \times 10^7$	6.38×10^7	$3.40 \times 10^7 – 1.62 \times 10^8$	4.07	8.65×10^7	$3.77 \times 10^7 – 3.73 \times 10^8$	5.51
Pantoea agglomerans	4.15×10^5	$2.67 \times 10^5 – 6.6 \times 10^5$	2.80×10^6	$1.20 \times 10^6 – 8.76 \times 10^6$	6.75	4.42×10^6	$1.97 \times 10^6 – 1.28 \times 10^7$	10.64

The LC_{50} dose of each bacterium for each aphid clone is shown along with lower and upper concentrations values at 95 % confidence limits. The calculated resistance factor for the two resistant aphid clones is also shown. *Resistance factor is a ratio of the LC_{50} value of the resistant clone to the LC_{50} value of the susceptible clone.

Table 3.6: Bioassay results with different bacteria against two different UK susceptible aphid clones.					
Aphid clones	**4106A (SUS-1)**		**4225B (SUS-2)**		
Bacteria	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**Resistance Factor***
Pseudomonas poae	1.1×10^2	$5.8 \times 10^1 – 1.92 \times 10^2$	3.3×10^2	$1.39 \times 10^2 – 6.49 \times 10^2$	0.64
Pseudomonas fluorescens	1.63×10^4	$1.08 \times 10^4 – 2.45 \times 10^4$	6.53×10^4	$6.48 \times 10^4 – 6.58 \times 10^4$	1.34
Pseudomonas jessenii	6.24×10^4	$2.72 \times 10^4 – 1.47 \times 10^5$	3.53×10^4	$1.64 \times 10^4 – 7.50 \times 10^4$	3.02
Citrobacter werkmanii	1.48×10^7	$9.27 \times 10^6 – 2.77 \times 10^7$	1.10×10^7	$7.56 \times 10^6 – 1.81 \times 10^7$	0.98
Escherichia albertii	2.92×10^6	$1.88 \times 10^6 – 4.91 \times 10^6$	9.04×10^6	$6.45 \times 10^6 – 1.42 \times 10^7$	1.38
Pantoea agglomerans	3.71×10^5	$2.04 \times 10^5 – 7.14 \times 10^5$	2.19×10^4	$7.15 \times 10^3 – 6.57 \times 10^4$	1.60
The LC_{50} dose of each bacterium for each aphid clone is shown along with lower and upper concentrations values at 95 % confidence limits. The calculated resistance factor for the two resistant aphid clones is also shown.					

Table 3.7: Bioassay results with different bacteria and two different susceptible aphid clones.					
Aphid clones	**4106A (SUS-1)**		**Clone-NS (SUS 3)**		
Bacteria	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**LC_{50} (Bacterial CFU mL^{-1})**	**95 % confidence limits**	**Resistance Factor***
Pseudomonas poae	1.1×10^2	$5.8 \times 10^1 – 1.92 \times 10^2$	6.9×10^1	$5.24 \times 10^1 – 9.00 \times 10^1$	0.63
Pseudomonas fluorescens	1.63×10^4	$1.08 \times 10^4 – 2.45 \times 10^4$	3.13×10^4	$1.56 \times 10^4 – 6.30 \times 10^4$	1.92
Pseudomonas jessenii	6.24×10^4	$2.72 \times 10^4 – 1.47 \times 10^5$	1.79×10^4	$6.51 \times 10^3 – 4.68 \times 10^4$	0.29
Citrobacter werkmanii	1.48×10^7	$9.27 \times 10^6 – 2.77 \times 10^7$	3.49×10^4	$2.08 \times 10^6 – 6.64 \times 10^6$	0.24
Escherichia albertii	2.92×10^6	$1.88 \times 10^6 – 4.91 \times 10^6$	2.47×10^6	$1.49 \times 10^6 – 4.56 \times 10^6$	0.85
Pantoea agglomerans	3.71×10^5	$2.04 \times 10^5 – 7.14 \times 10^5$	6.68×10^4	$3.17 \times 10^4 – 1.41 \times 10^5$	0.18
The LC_{50} dose of each bacterium for each aphid clone is shown along with lower and upper concentrations values at 95 % confidence limits. The calculated resistance factor for the two resistant aphid clones is also shown.					

3.2.4 Bacterial growth analysis

Data on the pathogenicity of six bacterial species against different insecticide and resistant aphid clones strongly suggested that at least some have potential as biocontrol agents. The oral toxicity against feeding on bacteria-treated diet indicated virulent bacteria have the ability to survive or replicate in sucrose rich diet and perhaps might survive on nutrient rich phloem sap and inside aphids during successful colonization. To examine the growth of bacteria, two different media named "LB media" (a routine bacterial growth medium) and Mittler diet, which mimics phloem sap composition, were considered. Growth curve analysis was performed using a Bioscreen plate reader with three replicates of the six bacterial species in LB medium and Mittler diet, at 20°C to mimic the similar growth conditions of aphids and plants.

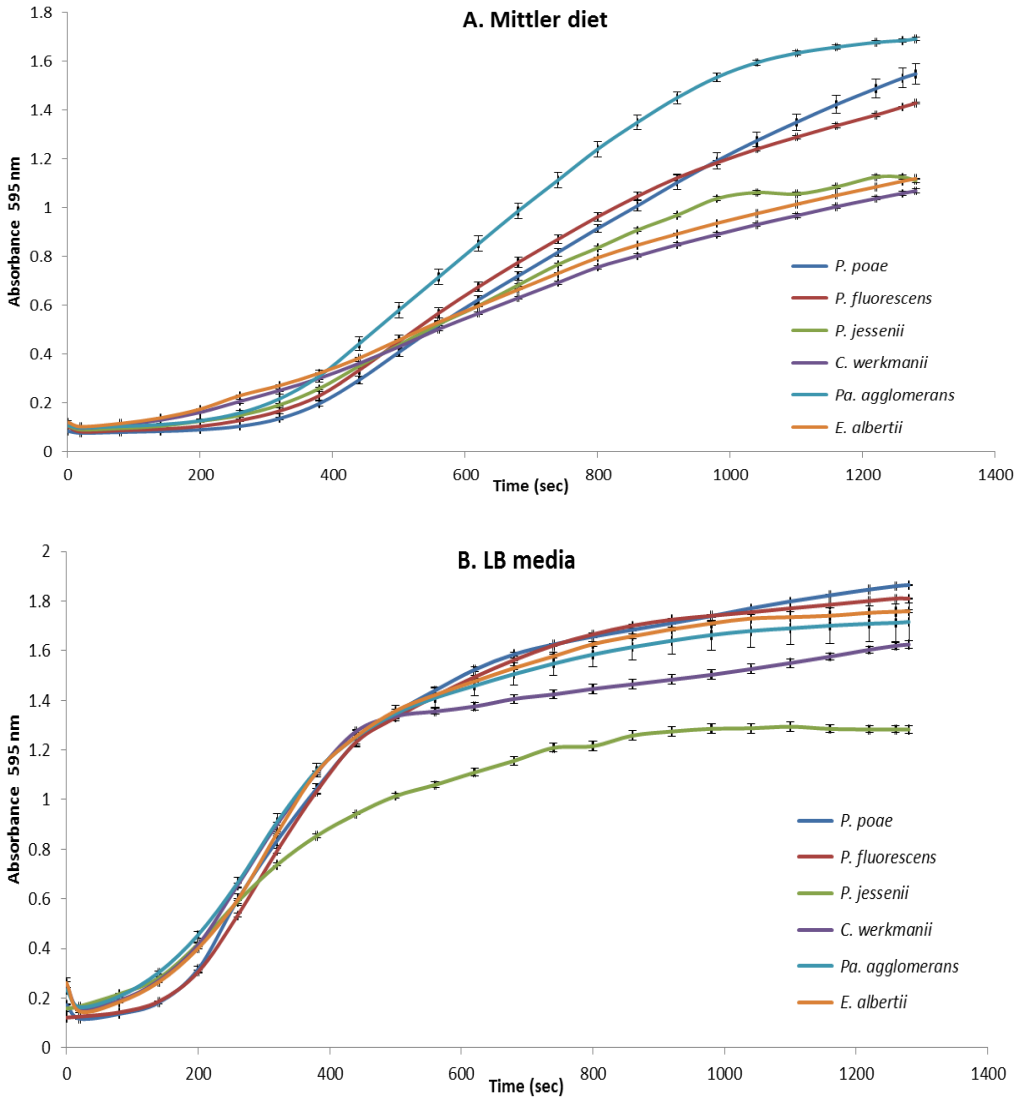


Figure 3.9: Growth curves of bacteria grown in different media. Bacteria were inoculated in to a 96 well microtiter plate and grown for 24 h in a plate reader at 20 ^{0}C. The data presented are the mean and standard error of three biological replicates.

Table 3.8: Summary of bacterial growth performance in different growth media.			
Growth rate of bacteria in LB media			
Bacterial Strain	**Vmax (mOD min $^{-1}$) (Maximum growth rate)**	**Standard error**	**Level of significance**
P. poae	3.952	0.063	A
P. fluorescens	4.071	0.086	A
P. jessenii	1.661	0.073	B
Pa. agglomerans	3.678	0.092	A
C. werkmanii	3.809	0.155	A
E. albertii	3.919	0.087	A
Growth rate of bacteria in Mittler diet			
Bacterial Strain	**Vmax (mOD min $^{-1}$) (Maximum growth rate**	**Standard error**	**Level of significance**
P. poae	1.48	0.02	B
P. fluorescens	1.47	0.04	B
Pa. agglomerans	1.94	0.05	A

In LB media, all bacteria exhibited a lag time of around 80 minutes probably due to a shift from their original growth conditions. After 120 mins, each strain started growing exponentially until they entered the stationary phase; and the final cell density reached more than 1.6 except in the case of *P. jessenii* where the final O.D. was 1.2. To assess differences in growth rate the Vmax (mOD min $^{-1}$) was calculated during the exponential phase of the growth curve. The results showed the *P. jessenii* Vmax was 1.6 mOD min $^{-1}$ significantly lower than the Vmax values of the other bacteria where no difference in growth rate of the five strains was observed (Figure 3.9 and Table 3.8).

In Mittler diet, a longer lag time of around 240 mins was observed in all bacterial species due to the shift from the previous LB growth media. After 9 h, only *P. poae, P. fluorescens* and *Pa. agglomerans* grew exponentially in Mittler diet reaching a maximum cell density of more than 1.5 in 24 h. However, the rest of the bacteria showed slower growth performance with a final cell density value around 1. Due to differences in growth rate, the Vmax was calculated on the three bacteria exhibiting the highest growth rate. Both *Pseudomonas* strains exhibited similar Vmax values whereas; *Pa. agglomerans* displayed a significantly higher Vmax as compared to other *Pseudomonas* strains (Figure 3.9 & Table 3.8).

In the growth analysis, *Pa. agglomerans* and two of the *Pseudomonas* strains were able to grow well in the nutrient rich Mittler diet which is thought to be analogous to the phloem sap composition, suggesting they might be able to grow on plant surfaces. Additionally, the aphid gut and hemolymph are composed of sugars and amino acids that support the growth of these bacteria (Wilkinson *et al.*, 1997; Cristofoletti *et al.*, 2003). It also implies that the bacteria may be able to replicate in the high sucrose concentration honey dew secreted by the aphids on plant surfaces. Hence, the presence of these virulent bacteria in "infected" honey dew secreted by aphids on plant surfaces might help to reduce the chances of another aphid infestation. This self-replenishing pesticide system could be utilised in pest management strategies.

However, reports of *Pa. agglomerans* pathogenicity towards mammals and plants exclude this species for further consideration as a biocontrol agent (Cruz *et al.*, 2007). Therefore, based on the aphid mortality results and growth performance in the diet, *P. poae* was selected as the bacterial species of choice on which to conduct further studies to understand the mechanism(s) of virulence against aphids.

3.2.5 Bacterial quantification in infected aphids

Aphid mortality upon bacterial challenge may result from toxic shock produced by the bacteria or alternatively could be due to massive bacterial growth within the aphids. Therefore, aphids were infected with the bacteria and macerated at six time points to release the microbial cells for culturing, which enabled accurate enumeration of bacteria during the course of infection.

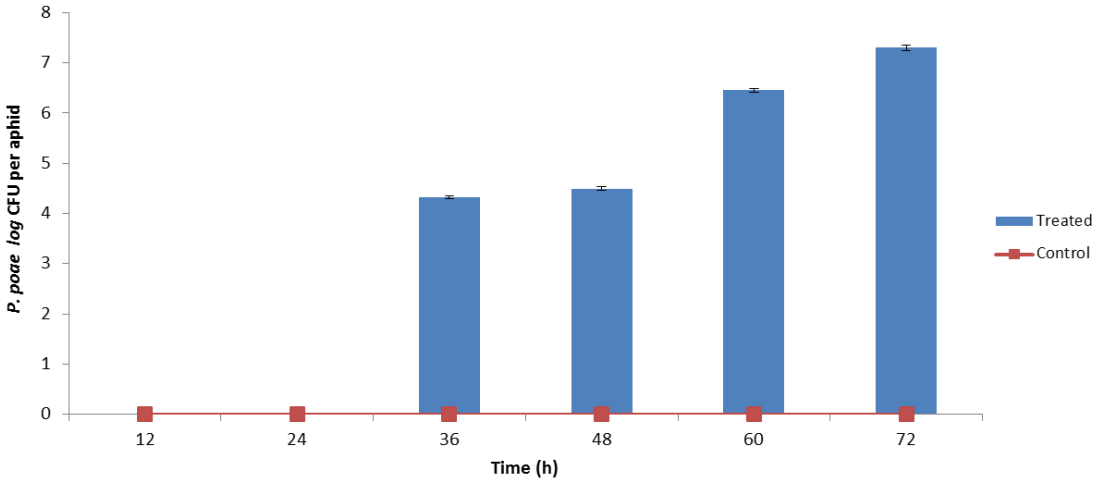


Figure 3.10: *P. poae* population growth inside aphid clone 4106A. *P. poae* populations within infected 4106A aphids were continually elevated to $2X10^7$ CFU/aphid over the period of inoculation and no colonies were recovered from control aphids for the entire duration of the experiment. **Control**: Ten aphids were fed in sterile diet with three replicates. **Treated**: Ten aphids, infected with 10^2 CFU mL^{-1} *P. poae* in sterile diet with three replicates. Error bars represent standard error of the mean.

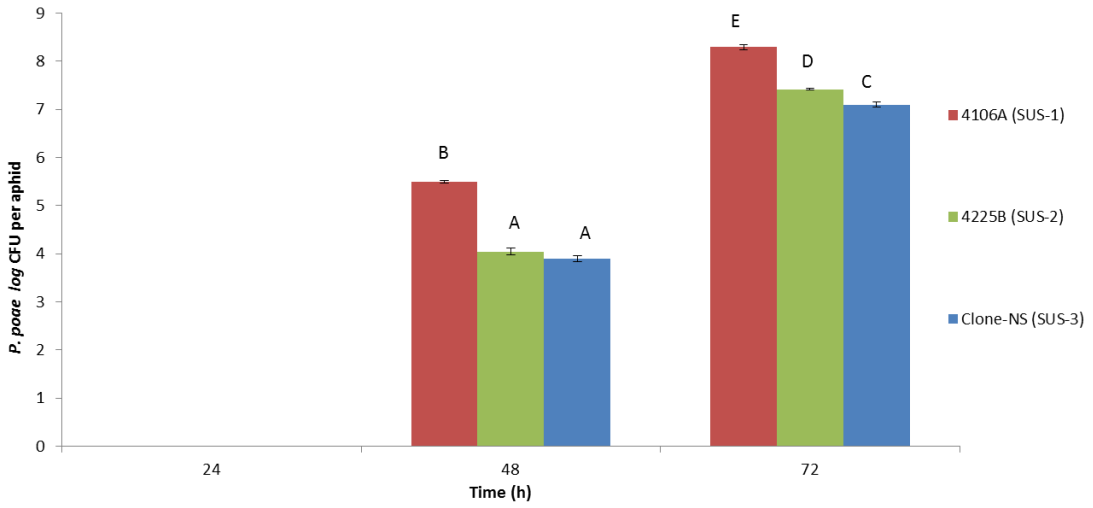


Figure 3.11: Assessment of *P. poae* population in all infected insecticide susceptible aphid clones. Mortality assay with inoculation dose of 10^2 CFU mL^{-1} on all sensitive clones for three days. After 48 h, *P. poae* CFUs of each aphid clone were determined by enumeration on LB-Nitrofurantoin plates. No colonies were recovered from control sachets. The data represent the mean and standard error of three biological replicates of *P. poae* treated sachets which contained ten aphids of each clone. The results show a statistically significant (different letter) decrease in CFUs of both 4225B and clone-NS as compared to 4106A clone ($p<0.05$).

The enumeration of *P. poae* within the 4106A aphid clone (SUS-1) was assessed every 12 h for three days with an inoculation dose of 10^2 CFU mL^{-1} in treated sachets. Until 24 h, *P. poae* bacteria were not recovered from infected aphids (Fig. 3.10). At 36 h, the *P. poae* titre reached to $2X10^4$ CFU/aphid and constantly increased to $2X10^7$ CFU/aphid till 72 h. No bacteria were recovered from the non-inoculated (control) aphid sachets.

Additionally, the comparative account of recovered *P. poae* bacteria from all infected insecticide-sensitive clones also revealed a higher susceptibility pattern in 4225B and Clone-NS compared to 4106A (Fig. 3.11). An assay was performed with an infection dose of 10^2 *P. poae* CFU mL^{-1} to monitor mortality and simultaneously record the *P. poae* population recovered from infected aphids at 24 h, 48 h and 72 h. During infection, no *P. poae* was recovered from any infected aphid clones until 24 h. At 48 h and 72 h, a lower titre of *P. poae* was observed in both 4225B and Clone-NS as compared to the reference 4106A susceptible clone, which were statistically significant (*$p<0.05$) at their respective time points (Fig. 3.11). Furthermore, mortality assays showed no death in any aphid clones until 48h. After 72 h, the maximum mortality rate (i.e. 60 %, Figure 3.4 E) was observed in Clone-NS followed by 45 % death in 4225B (Figure 3.4 F) and only 16 % death reported in 4106A (Figure 3.4 G). These data indicate lower resistance (i.e. more sensitivity towards bacteria) for Clone-NS and 4225B as compared to 4106A aphid clone.

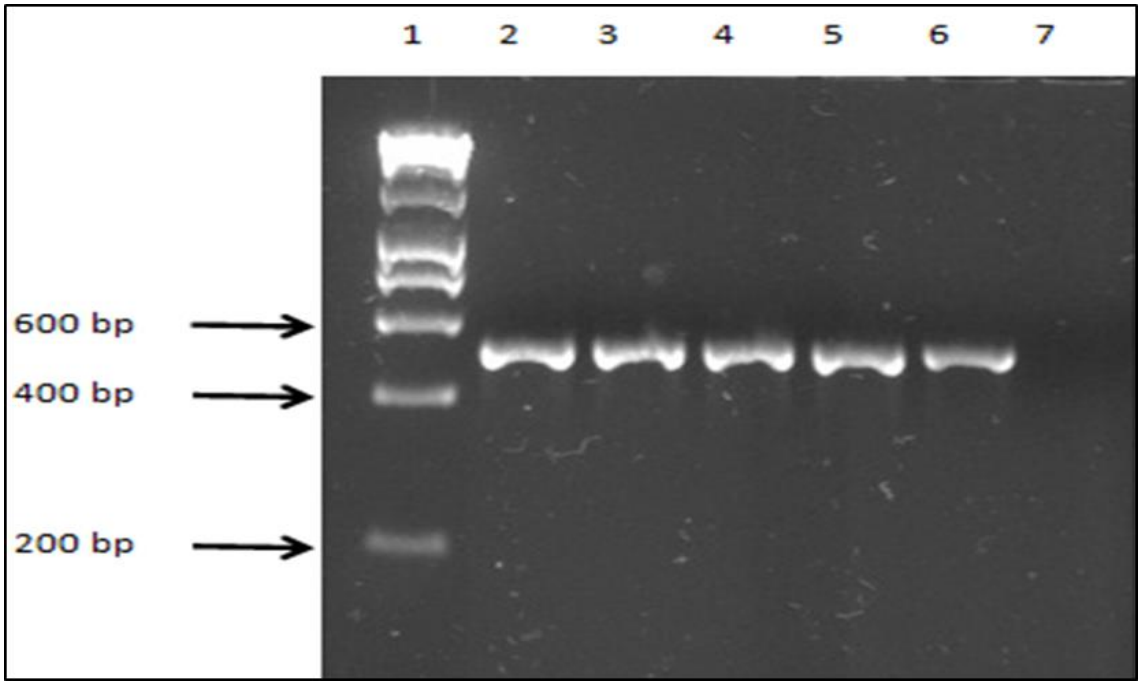


Figure 3.12: Amplification of the *tcaA* gene from *P. poae* recovered from aphids.
DNA was electrophoresed through a 1 % agarose gel for 60 min at 80 volts. The 500bp TcaA amplicon was observed at all-time points. Lane 1 – Hyper Ladder I; Lane 2 to 5 – 36 h, 48 h, 60 h & 72 h bacteria culture; Lane 6 – *P. poae* DNA (positive control); Lane 7 – (Negative control) no DNA.

Furthermore, to confirm whether the recovered bacteria from infected aphids was *P. poae*, a PCR was performed on single colonies of bacteria recovered at each time point (from 36 h to 72 h) using specific TcaAF1 and R1 primers to amplify the *P. poae* TcaA toxin gene. All colonies tested led to amplification of the toxin gene indicating recovery of *P. poae*.

These results indicate that even consumption of low doses of bacterial cells may be enough to cause death of aphids over time if they successfully replicate before being eradicated by the aphids immune system.

3.2.6 Effect of bacterial challenge on aphid fecundity

Another aim of this chapter was to monitor the fecundity (number of nymphs produced) by the aphids when fed on both control and bacteria-treated diet. The importance of measuring fecundity was to assess whether the artificial diet ingredients provide proper growth and support to enable normal reproduction and, importantly, to assess if bacterial challenge also had effects on reproduction. Reproductive fitness was assessed in all resistant and susceptible clones on exposure of bacteria challenge.

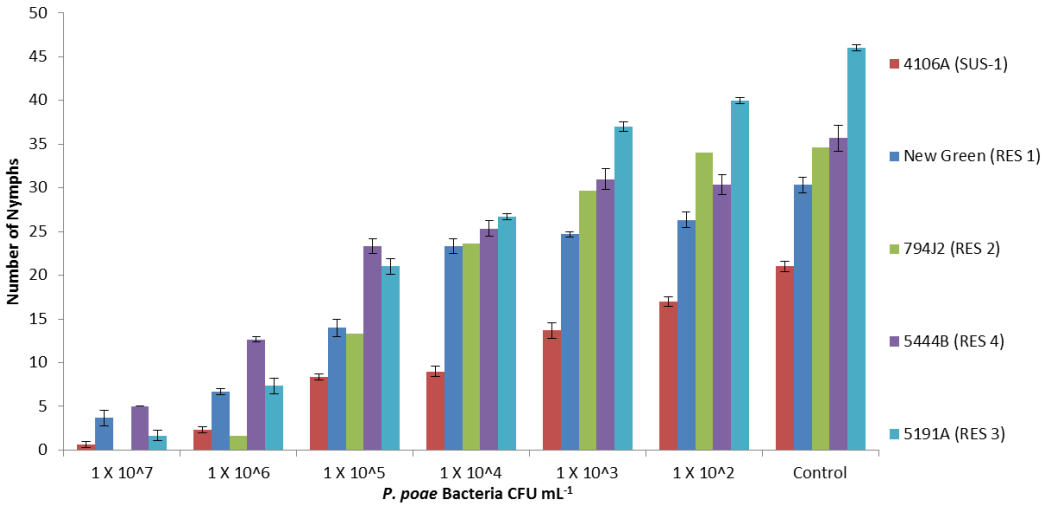


Figure 3.13: Influence of *P. poae* challenge on the fecundity of all five aphid clones. Columns represent the number of nymphs produced at 72 h in bacterial-treated at different infection doses and control aphid sachets of all insecticide resistant and susceptible aphid clones. Error bars represent standard error of the mean.

Table 3.9: Summary of two way ANOVA-HSD test.				
Source	**Degrees of Freedom (DF)**	**Sum of Squares**	**Mean sum of squares**	**Prob > F**
Aphid clone	4	2914.152	728.538	<.001
Bacteria	6	12772.46	2128.743	<.001
Aphid clone*Bacteria	24	1234.114	51.421	<.001
Residual	70	123.333	1.762	
Total	104	17044.06		

Table 3.10: Statistical similarities and differences between nymph productions by different aphid clones in *P. poae* treated diet.

Aphid clone. Bacteria Dose	Mean value of Nymphs	Significant letter
794J2 10^7	0	A
4106A 10^7	0.67	A
5191A 10^7	1.67	A,B
794J2 10^6	1.67	A,B
4106A 10^6	2.33	A,B
New green 10^7	3.67	A,B,C
5444B 10^7	5	B,C,D
New green 10^6	6.67	C,D
5191A 10^6	7.33	C,D
4106A 10^5	8.33	D
4106A 10^4	9	D,E
5444B 10^6	12.67	E,F
794J2 10^5	13.33	F,G
4106A 10^3	13.67	F,G
New green 10^5	14	F,G
4106A 10^2	17	G,H
4106A Control	21	H,I
5191A 10^5	21	H,I
5444B 10^5	23.33	I,J
New green 10^4	23.33	I,J
794J2 10^4	23.67	I,J
New green 10^3	24.67	I,J
5444B 10^4	25.33	J
New green 10^2	26.33	J,K
5191A 10^4	26.67	J,K
794J2 10^3	29.67	K,L
5444B 10^2	30.33	K,L,M
New green Control	30.33	K,L,M
5444B 10^3	31	L,M,N
794J2 10^2	34	M,N,O
794J2 Control	34.67	N,O
5444B Control	35.67	O
5191A 10^3	37	O,P
5191A 10^2	40	P
5191A Control	46	Q

The test performed was a Two factor (Aphid clone and bacteria dose) ANOVA followed by Tukey-Kramer HSD shown as different letters indicate statistically significant differences. Colour Coding cells - Red –Insecticide susceptible & Blue-Insecticide resistant.

To measure fecundity, ten aphids of each clone were transferred to three biological *P. poae* treated sachets and control sachets. After 72 h, the number of nymphs produced in both *P. poae*-treated and a control aphid sachet was assessed.

A higher number of nymphs were observed from all aphid resistant clone control sachets ranging from 46-36 and the lowest value 21 was observed in the aphid susceptible clone control sachet, which showed the differences in the fecundity of the aphid clones when fed on diet. When fed on *P. poae*, at higher concentrations (10^6-10^7 CFU mL^{-1}) a negligible amount of nymph production was observed in all aphid clones with the exception of the two resistant clones 794J2 & 5444B, where 10-12 nymphs were oberved at this bacterial concentration. In contrast, nymph production was observed at a moderate rate when fed on bacterial concentrations ranging between 10^2 to 10^5 CFU mL^{-1} in all aphid clones.

The statistical test (two way ANOVA-HSD test) defined all control sachets (without bacteria) for each aphid clone was significantly different than the bacteria-treated sachets ($p<0.001$) (Table 3.9). Nymph production in the higher bacterial concentration (10^6-10^7 CFU mL^{-1}) sachets showed highly significant differences between different aphid clones ($p<0.001$) (Table 3.10). Conversely, lower concentrations ranging between 10^2 to 10^5 CFU mL^{-1} showed all aphid clones reproduced at a similar rate with no significant differences (*$p<0.001$) (Table 3.10).

This indicates that higher bacterial cell concentrations are both efficient at killing *M. persicae* and reduces its rate of reproduction.

3.3 Discussion

Insecticide resistance in aphids presents a major constraint on our ability to protect the yield and quality of a number of important crop plants. Because there are only a limited number of insecticides with different modes of action available, and on-going legislation are likely to further limit the number of compounds in the insecticide arsenal there is an urgent need to develop alternative control strategies. In this context, the interactions between insects, such as aphids, and microorganisms could be of crucial importance as it could lead to the discovery of biological molecules that can be used for the control of insects, as in the case of *B. thuringiensis* crystal proteins and many insect larvae (Schnepf *et al.,* 1998).There has been evidence of epiphytic bacteria that colonize the surface of plants and that can be phytopathogenic such as *Erwinia aphidicola* (Harada & Ishikawa, 1997), *P. syringae* pv. *Syringae* (Stavrinides *et al.,* 2010), *Pantoea stewartii* (Stavrinides *et al.,* 2009) and *D. dadantii* (Grenier *et al.,* 2006), which are both phytopathogenic and entomopathogenic, active in particular against the pea aphid. These bacteria that are phytopathogenic, are thought to have initially exploited insects as vectors and over time have evolved a novel mode of interaction with insects, retaining an ability to colonize them and use them as secondary hosts (Nadarasah & Stavrinides, 2011).

Recent study has identified 14 plant-residing bacteria which were found to be pathogenic to aphids (Livermore, 2016). The results revealed that the bacteria have a variable degree of pathogenicity toward six different aphid species during a three day course of infection (Livermore, 2016). Because insecticides are used so intensively to control aphids, and that resistance is such a significant and growing problem, it was particularly important to establish the efficacy of any potential biocontrol on insecticide resistant strains or clones of aphids. Hence, the first step was to screen a range of bacteria on different insecticide-resistant aphids to investigate any relationship between bacterial susceptibility and insecticide resistance. An artificial feeding system with a liquid diet was used as a high-throughput screening system to identify pathogenic bacteria against aphid *M. persicae* (“wild type” susceptible clone plus

insecticide resistant clones) (refer to section 2.5) (Dadd *et al.,* 1967). Next, ten phylloplane bacteria were added to the artificial diet and aphid mortality tests were conducted at different concentrations. As a result of these assays, six bacterial species, tentatively identified as *P. poae*, *Pa. agglomerans*, *P. fluorescens*, *P. jessenii*, *C. werkmanii* and *E. albertii* were shown to be pathogenic to all aphid clones. Analysis of aphid toxicity assays at all-time periods suggested that none of the bacterial species caused aphid mortality at 24 h. At 48 h all bacterial strains except *Pseudomonas jessenii* showed exhibited signs of aphid killing (Figure 3.3 A-G). After 72 h, all bacterial strains caused high levels of aphid mortality (Figure 3.4 A-G). These results are similar to the findings seen for *Dickeya dadantii* A428 strain and other enteric bacteria, which resulted in 50 % to 100 % aphid mortality after 4–5 days of ingestion of bacteria through the diet (Grenier *et al.,* 2006). Interestingly *P. fluorescens* and *Pa. agglomerans* were previously identified as potential pathogens for *M. persicae* (Hashimoto, 2002). Recently, the genome sequencing of *P. poae*, *C. werkmanii* and *P. fluorescens* revealed genes encoding potential insecticidal protein toxins in these bacteria, correlating with their pathogenicity to aphids (Livermore, 2016). There are a variety of killing mechanisms that have been reported in insect pathogenic bacteria, such as *Pantoea stewartii* DC28, where the bacteria aggregates in the aphid gut and hinders the flow of honeydew and excretion (Stavrinides *et al.,* 2010). *P. fluorescens* Pf-5, however, harbours a gene encoding a large protein toxin "Fit", which exhibits insecticidal activity (Péchy-Tarr *et al.,* 2008). The latter provides evidence of potential toxins that may be secreted from bacterial strains, which are included in this study.

Insecticide resistance in *M. persicae* is an evolutionary adaptation that can produce mechanisms with associated fitness costs in the absence of insecticides (Foster *et al.,* 1997a, 2000b, 2003c). In the current study, aphid mortality and fecundity tests were used to examine all insecticide resistant and susceptible aphid clones to investigate if resistant aphids are more or less fit to bacterial challenge. Fecundity tests indicated higher nymph production by all insecticide resistant aphid clones compared to susceptible clone on Mittler diet with and

without bacteria. These findings are similar to those previously reporting that *M. persicae* insecticide-resistant clone's growth rates vary but can be equal or greater than fully susceptible clones (Fenton *et al.,* 2010). Further studies are needed to understand the genetic basis of the observed differences in fecundity between strains but in the context of this study there was no observable fitness penalty in terms of reproductive output associated with resistance when challenged with bacteria. As expected, the current study revealed that nymph production was significantly lower in bacterial-treated sachets than controls and, as the bacterial concentration increased nymph production decreased significantly. These results support recent work showing infection of *P. syringae* B728a caused mortality to aphids, although they contrast with the description of elevated aphid reproduction with increasing dose of *P. syringae* B728a ranging from 10^2 to 10^7 CFU mL^{-1}. High doses of this strain led to high mortality and very little aphid reproduction (Hendry *et al.,* 2016). This outcome suggested increments of aphid reproduction occurred with increasing bacteria dose promotes fecundity compensation or investment in reproduction rather than immune response to a pathogen.In the current study, nymph production was recorded after 72 h in in aphid sachets with and without bacteria in the artificial diet, which is an artificial system. Hence, future aphid fecundity assay should be performed where orally infected aphids are exposed to varying bacterial doses of *P. poae* and then their survival, development time, and reproduction rates assessed on healthy plants.

The current work identified *P. poae* as the most pathogenic bacteria to all aphid clones. This statement can be supported with the better growth performance of *P. poae* in sucrose rich Mittler diet as compared to the growth curves of other bacteria. Additionally, *P. poae* growth analysis inside aphids revealed a continually increased bacterial load over the period of infection that supported successful colonization inside aphids. These results confirmed former studies of two pathogenic *Pseudomonas* strains (*P. entomophila* L48 *and P. syringae* B728a), which efficiently colonize and multiply inside the insect digestive tract and kill insects. In this study *P. fluorescens* was more toxic to 5191A and New green followed *by C. werkmanii and E.*

albertii. Pa. agglomerans was more pathogenic to New green and only *P. jessenii* was found to be more effective in killing 5444B and New green. A similarly low infective dose, around 100 cells, was also found for the *P. syringae* strains to kill pea aphids, so this may be a trend common to other plant-associated bacteria that cause death in hemipteran insects (Hendry *et al.,* 2016; Stavrinides *et al.,* 2009).

The aphid susceptible clone 4106A has been used as a standard control to measure baseline susceptibility or relative resistance of resistant clones for each bacterial challenge. However, due to differences in physical parameters such as water content, humidity and light source at the different labs (Reading University & Rothamsted research insectary) variation in LC_{50} values of 4106A clone was observed (Table 3.4 & 3.5). Additionally, another UK origin susceptible clone 4225B showed similar LC_{50} values as 4106A clone for all the different bacteria challenges with the exception of *P. poae,* and provide further evidence that there is no consistent correlation of insecticide resistance status and susceptibility to bacterial challenge. In contrast, an additional susceptible Clone-NS showed large variation in LC_{50} values as compared to 4106A clone suggesting the genetic background is a more important factor in bacterial sensitivity than insecticide resistance status. Enumeration of *P. poae* cells was done in infected susceptible aphid clones and linking this to mortality rates shed some light on how pathogenic bacteria load could correlate with intrinsic susceptibility of different aphid genotypes. Overall, in the analysis of three insecticide susceptible clones and four resistant clones, a significant variation was observed in response to bacterial challenge and no consistent trend was observed between insecticide resistance status and susceptibility to all bacterial species tested. No single bacterial strain was identified that was consistently more toxic to insecticide resistant clones than susceptible clones suggesting there is no penalty in resistant clones (as a result of modified nervous system proteins or overproduction of enzymes) that makes such clones less fit to bacterial challenge. However, further screening of a larger number of aphid genotypes carrying different resistance mechanisms should be conducted to check that this finding was not influenced by the relatively small sample size employed in this study.

In contrast, the two most-insecticide resistant clones, 5444B and 5191A, showed moderate levels of tolerance to *E. albertii, Pa. agglomerans & C. werkmanii* with up to ~11 fold resistance observed compared to the susceptible reference clone (Table 3.5). Furthermore, the most insecticide resistant clone, 5444B, showed low to moderate resistance (4-11-fold) to five of the six bacterial species tested. Although these findings are consistent with the hypothesis that enhanced production of detoxification enzymes in these aphid clones (or altered insecticide target sites) provides cross-resistance to bacterial challenge, it is also possible that other genes that have no involvement in insecticide resistance confer resistance to bacterial challenge in clones 5191A and 5444B. In the case of 5191A this clone has adapted to feed on tobacco and overcome the toxic plant secondary metabolite nicotine (Bass *et al.,* 2013) and host adaptation may also play a role in the intrinsic susceptibility of certain aphid clones to bacterial challenge. In resistant aphid clones, higher expression of major detoxifying genes (such as esterases, glutathione S-transferase, cytochrome P450 and others) against allelochemicals might be utilised against bacteria pathogen attack. Regardless of the mechanisms underlying bacterial resistance current findings are important as they demonstrate that 1) different aphid genotypes show different susceptibility to bacterial challenge and the application rates of any biological control based on the deployment of these bacteria or their toxin(s) would need to take this into account, 2) the level of resistance observed <11-fold are relatively modest and application rates could be devised that would still ensure good efficacy against insecticide resistant aphid clones. The latter point means that such strains used as biological control would be 'resistance busting' and would provide an invaluable control option against populations of *M. persicae* that can no longer be controlled with most conventional insecticides.

In conclusion, *P. poae* was identified as the most potent bacterium to kill all insecticide susceptible and resistant aphid clones therefore, potential insecticidal protein toxins in this bacterium, correlating with their pathogenicity to aphids, should be investigated further. Furthermore, the efficacy of *P. poae in planta* needs to be tested; thus, experiments will be

conducted to examine bacteria longevity on plants, and aphid mortality and fecundity on untreated plants compared to plants inoculated with this aphid killing bacterium.

4 *Pseudomonas poae* colonisation on plants

4.1 Introduction

Among biotic stresses, plant eating insects and pathogenic microorganisms are a serious threat to crop production and ecosystem stability. Currently, the number of pesticides in practice to manage pest infestation are being utilised depending on the time of harvesting crops and severity of infestation. Traditional chemical pesticides usually have one of 3 modes of action, systemic, trans-laminar or contact (Sanderson, 2011). Systemic pesticides are absorbed by plants relatively quicker via the roots or above-ground plant tissues, and are then circulated within the vascular system. Trans-laminar pesticides referred to as "local systemic" are applied directly where the pest is located, or they need to be circulated uniformly over the plant surfaces from which the pest are likely to feed on them. Contact pesticides generally control a pest as a result of direct contact. To make efficient contact with the target, contact pesticides should be applied with excellent coverage of spray droplet. Systemic or translaminar pesticides tend to be more effective than contact pesticides, provided that a sufficient amount of pesticide reaches the aphid feeding sites, but chewing insects may not get enough to be controlled (Sanderson, 2011). However, in the case of contact pesticides, typically two applications of foliar sprays, a week apart, are often needed to ensure maximum benefit. As these pesticides require direct contact of pests, if the pests are protected by being on the underside of leaves, or by dense foliage, they may not work to their optimum capability. The other reasons for pesticide ineffectiveness are resistance to chemicals and its limitation on specific growth stages of the insect's generally small size of larva. It has been reported in cereal leaf beetle (*Oulema melanopus*) that application of pesticides are more effective when small rather than large larvae and adults are present (Hines, 2001). Besides these, most larva of beetles, moth and flies are situated in the stem of the plants or in the soil, and due to inadequate coverage of spray in these regions, this can lead to a resurgence of pest numbers after maturation of these concealed larvae (Hines, 2001). Moreover, the use of conventional agrochemicals can cause severe effects on environment, and short- and long-term human health issues (Damalas & Eleftherohorinos, 2011).

A biological control, where a natural enemy is released into the pest's environment, is a popular way of dealing with pest infestations. This strategy is usually cost effective in controlled glasshouses however; the outbreak of short generation time pests like aphids can easily exceed the predator population resulting in detrimental infestation. Hence, the use of chemical pesticides, following established guidelines, can sometimes be part of control measures with the goal of removing only the target pest.

To address problems raised by use of conventional and biological pesticides, microbial pesticide has been introduced in pest management. Currently, the use of microbial pesticides includes entomopathogenic microorganisms (and sometimes includes the metabolites that bacteria or fungi produce) with improved formulation methods that have been employed. The bacteria recovered from disease suppression soils and the plant phylloplane and rhizosphere are likely to be used as novel bio-control agents. The work suggested that an efficient biocontrol strategy involving direct antagonism mechanisms of indigenous phylloplane bacteria would be useful to maintain pathogen populations at low levels (Halfeld-Vieira *et al.,* 2015). From this perspective, native phylloplane microorganisms could play this role through utilization of carbon and nitrogen sources on leaves to establish and maintain their own population (Wilson & Lindow, 1994a; Wilson & Lindow, 1994b; Lindow, 2000; Smith & Lindow, 2013). The proactive competition between the antagonist and the pest due to nutritional similarity for carbon and organic nitrogen sources led to reduced populations of pest (Dianese *et al.,* 2003). Secondly, the antagonist could hinder pest growth by secreting antibiotics and other secondary metabolites, thus accounting as another mode of control mechanism. Besides the mechanisms that inhibit the pathogen population establishment on leaves, plant associated bacteria have the ability to trigger induced systemic resistance (ISR) prior to infection by a pathogen (Vieira *et al*., 2006; Romeiro *et al.,* 2010). In this scenario, although the pathogen is able to maintain a minimal population to cause infection, the induced resistance against the pathogen results in low levels of disease development t (Conrath *et al.,* 2002).

Nowadays, new research indicates that, besides the more well-known entomopathogenic microorganisms like *B. thuringiensis,* other soil-living organisms such as fluorescent pseudomonads and the nematode-associated bacteria, *Photorhabdus spp.* and *Xenorhabdus spp.,* carry genes encoding for insecticidal secondary metabolites (Duchaud *et al.,* 2003; Vodovar *et al.*, 2005; Challacombe *et al.,* 2007; Olcott *et al.,* 2010; Waterfield *et al.,*2016). Plant growth-promoting rhizobacteria (PGPR) are known to trigger ISR in plants and restrict establishment of infection by the pathogens in the host (van Peer *et al.,* 1991; Gang Wei *et al.,*1991). *Pseudomonas spp.* are known to protect plants from pathogens through various mechanisms, viz., ISR in the host (Van Peer *et al.,* 1991; Maurhofer *et al.,* 1994), antibiotic production (Maurhofer *et al.,*1995), growth promotion (Schippers *et al.,* 1987), and competition for nutrients (Duijff *et al.,* 1993; Leeman *et al*., 1996). These characteristics make *Pseudomonas* species good candidates for using as seed inoculants and root dips for biological control of plant pathogen. Several *Pseudomonas* species such as *P. protegens* CHA0*, P. fluorescens, P. brassicacearum* and *P. chlororaphis* are very potent biocontrol agents against

plant-pathogenic bacteria, fungi and insects (Stutz *et al.,* 1986; Meena & Marimuthu, 1995; Otsu *et al.,* 2004; De Werra *et al.,* 2009; Zhou *et al*., 2012; Corrêa *et al.,* 2015).

Plant associated bacteria can promote plant growth and development directly by producing or degrading plant hormones or modifying phytohormonal signalling pathways. Indole-3-acetic acid (IAA) production genes were reported in *P. chlororaphis* O6 and genes for catabolism of the plant hormone were found in *P. fluorescens s*trains 30-84, O6, and Pf-5 (Wightman & Douglas, 1982; Kim *et al.,* 2004; Dimkpa *et al.,* 2012).Previous studies have shown that *P. fluorescens* F113 is able to mobilize insoluble soil phosphate into soluble bioactive forms that can be taken up by plant roots (Miller *et al.,* 2010; Rice *et al.,* 2012). Aminocyclopropane-1-carboxylic acid (ACC) deaminase producing root associated bacteria (e.g. strains *P. fluorescens* Q8r1-96 *&* F113*)* reduce ethylene levels by converting ACC into ammonia and α-ketobutyrate, thus promoting root growth and improving tolerance to abiotic and biotic stress (Loper *et al.*, 2012; Redondo-nieto *et al.,* 2013). These bacteria can also contribute to the promotion of plant growth by emitting Volatile organic compounds (VOCs). A recent study demonstrated that *P. fluorescens* SS101 promotes plant growth via the release of VOCs including 13-Tetradecadien-1-ol, 2-butanone and 2-Methyl-n-1-tridecene (Park *et al.,* 2015). Additionally, the application of VOCs can mediate induced resistance in cucumber plants against both the bacterial angular leaf spot pathogen, *P. syringae pv. lachrymans*, and the sucking insect aphid, *Myzus persicae*. These results demonstrate that VOCs may help prevent plant disease and insect damage by eliciting induced resistance (Song & Ryu, 2013).

Several reports on root associated microbes showed ability to induce plant resistance in systemic tissues by interfering with different plant signalling pathways. It has been described that signalling pathways that regulate ISR and plant defences against herbivores are partly interrelated (Van Oosten *et al.*, 2008; Van Wees *et al.*, 2008; Van de Mortel *et al.*, 2012). The application of *P. fluorescens* WCS417r strain on Arabidopsis roots induced resistance to herbivore attacker via Jasmonic acid (JA) and Ethylene (ET) dependent signalling pathways, while *P. fluorescens* SS101 mediated resistance through the salicylic acid (SA) pathway and induced secondary metabolite (glucosinolate and camalexin) biosynthesis (Pieterse *et al.*, 1998; Van de Mortel *et al.*, 2012). These examples suggested that plant associated *Pseudomonas* act via different phyto-hormonal signalling pathways that enhance plant defence to either pathogens or insect herbivores.

Besides these, the excellent efficacy of various *Pseudomonas* strains to control pathogens by different application methods, including foliar, soil, seed treatment and drip irrigation have been well studied in potato, apple, rice and several other crops (Meena & Marimuthu, 1995;

Mohsin Tariq *et al.,* 2010). Thus, microbial pesticides are a newly emerging method of biological control as they are cost effective and environmentally safe. The reliability of any novel microbial pesticides would be evaluated by the features listed below:

1. Any deleterious effect on plant growth and size.
2. Effect on natural populations on plant surfaces
3. Any influence on natural pollinators or beneficial insects
4. Survival rate or longevity on the plants along with any additional nutritional requirements.
5. Should be nontoxic to human health and other animals

All these features should have maximum score for effective formulation of microbial pesticides. Hence, the ideal plant-bacteria interaction is of the approach which would help to introduce novel pesticides for sustainable crop protection method.

In my study, six bacterial strains isolated from environmental sources were classified as 50 % - 100 % pathogenic in an *in vitro* aphid killing assay. To further evaluate the bacterial interaction with plants and aphid, *in vivo* experiments were carried out. The previous mortality assay in this study revealed the most potent bacterium *P. poae* can kill aphids in 48 h when inoculated in artificial Mittler diet. Considering the application method, a surface spray methodology was mostly used to attract pest on surface coated with a deleterious substance (toxin or pathogen) (Foster & Harris, 1997), thus the bacteria must be able to survive, and be ingested by the aphids, from the surface of the plant. To achieve reduced aphid populations, investigations would be carried out to test pathogenicity of bacteria to all growth stages of aphids and substantial amount of bacteria on plants with longer shelf life.

In addition to *P. poae* oral toxicity towards aphids, any volatile organic compounds released by this beneficial bacterium might attract or repel insects. It has been demonstrated that plant beneficial *Pseudomonas* strains can induce resistance in the plants against herbivore or pathogen attack through JA, ET and SA signalling pathway. All these phytohormonal signalling pathways are involved in secondary metabolites and green leaf volatiles, which provide direct and indirect plant defences. Interestingly, the effect of beneficial microbes on the emission of green leaf volatiles are variable, where it has more often shown increased emission of the terpenoids or HIPVs (Pineda *et al.,* 2013) and only once shown suppressed emission of HIPVs (Fontana *et al.,* 2009). Therefore, I hypothesized that *P. poae* can modify plant physiology by releasing a specific blend of green leaf volatiles and interfering with the phytohormonal signalling pathways. These modified leaf volatiles could potentially have positive or negative

effects on aphid performance. Hence, it is worth investigating the altered behaviour of aphids after application of *P. poae* spray on plants.

Considering all these aspects, *in planta* assays were carried out to determine:

1. The survival rate or longevity of *P. poae* on the tested plants after foliar spray or seed treatment method.
2. The effect of *P. poae* colonisation on aphid populations.
3. The efficacy of *P. poae* to control aphid populations over time.
4. Deterrence behaviour of bacteria-treated plants on aphids by olfactometer assay and choice experiments.

4.2 Results

4.2.1 *P. poae* survival *in planta*

To investigate how bacteria can adapt to various carbon and organic nitrogen composition of plant surfaces, a bacterial colonisation assay was conducted to examine survival rate of bacteria on three different plants: :

1. Plant Model "*Arabidopsis thaliana"* (Col-0 ecotype),
2. *Beta vulgaris* (Sugar beet, agricultural crop)
3. *Capsicum annum* (Pepper, horticultural crop)

Both infiltration and spray application methods were used to introduce bacteria on *Arabidopsis thaliana* (Col-0) leaves in the preliminary plant bacteria colonisation assay. I observed almost the same log CFU of phylloplane bacteria recovered from both methods at day of inoculation. After an initial drop in bacterial populations in the first 24 h, from day 1, the bacterial populations recovered from sprayed Col-0 leaves to a significantly higher population than those recovered from the bacterial infiltration method, during a course of experiment (Figure 4.1). No bacteria were recovered from non-inoculated plants. These results helped me to focus on using the foliar spray method for further assays. This was also helpful because the foliar spray method was less time consuming and it provides a more realistic approach to spray equal volume of bacteria CFU mL^{-1} on the both sides of leaves through use of hand atomiser. Additionally, similar numbers of bacteria was recovered from plants when bacterial cells were either suspended in PBS solution (pH=7.4) or sterile water. This suggested a versatile ability of the *Pseudomonas* strain to survive in diverse conditions. PBS solution acts as isotonic and non-toxic to cells therefore for subsequent work bacterial suspension made in PBS solution.

In the next step, bacterial enumeration was carried out on *Arabidopsis* (Col-0) and other two economically important crops; peppers & sugar beet. Colonisation was assessed at six time points: 0, 1, 3, 7, 14 and 21 days. At each time point the whole leaf was aseptically excised and processed as described in section 2.7.1. Bacterial counts obtained for each time point represent total counts, i.e. both for surface-adhering bacteria and any internalised cells. The highest bacterial populations were recovered at day 3 from leaves of all the plant species tested (except sugar beet, which had a slightly higher population at 21 days). The populations remained stable over the period of three weeks of colonisation (Figure 4.2). The data from all time points were analysed for differences between the counts for each of the plant species tested by one-way ANOVA, GenStat 16.0 for Windows (VSN International Ltd, Hemel Hempstead, and U.K). *P. poae* colonisation on the leaves of *C. annum* showed higher numbers

of bacteria were recovered after foliar spray inoculations as compared to other two tested plant species after three days, although statistical tests showed that this difference was not significant (*p value >0.05).

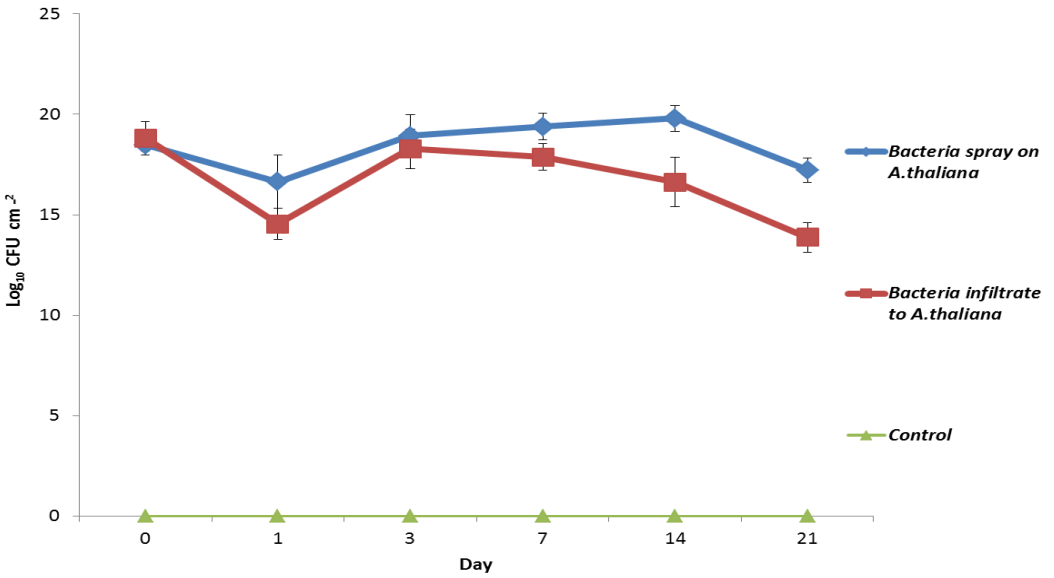


Figure 4.1: *P. poae* colonisation on *A. thaliana.* Bacterial populations recovered from Col-0 leaves after spraying and infiltration with cell suspension of 10^7 CFU mL^{-1} over a period of 21 days. For foliar spray, bacteria were suspended in sterile PBS solution and a leaf disc was collected at all time points. Each leaf disc was homogenised in PBS solution and serial dilutions were plated on LB with nitrofurantoin to count bacterial populations. The data presented are the mean and standard error of six biological replicates.

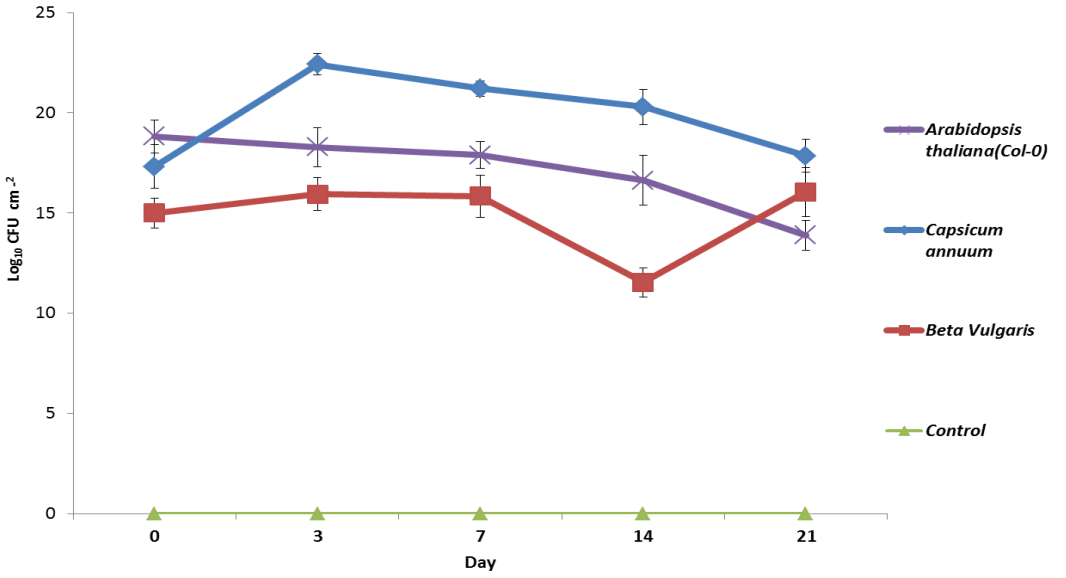


Figure 4.2: *P. poae* survival on three different plant species. Bacterial populations were recovered from plant leaf surfaces after spraying with cell suspension of 10^7 CFU mL^{-1} over period of 21 days. For the foliar spray, bacteria were suspended in sterile PBS solution and a leaf disc was collected at all time points. Each leaf disc was homogenised in PBS solution and serial dilutions were plated on LB with nitrofurantoin to count bacterial populations. The data presented are the mean and standard error of six biological replicates.

In all cases, it was possible to isolate *P. poae* from each of the plant species tested, showing it is able to survive in this environment for the duration of this test, albeit there is a large amount of variation between plant species that appears to be affecting the ability of *P. poae* to persist on the leaves. Besides this, I did not observe any hypersensitive reactions on any tested plants during the observation (Figure 4.3).

I also employed a seed soak method to apply *P. poae* bacteria on the pepper seeds but I failed to recover *P. poae* bacteria from treated seeds at all time points except the day of inoculation.

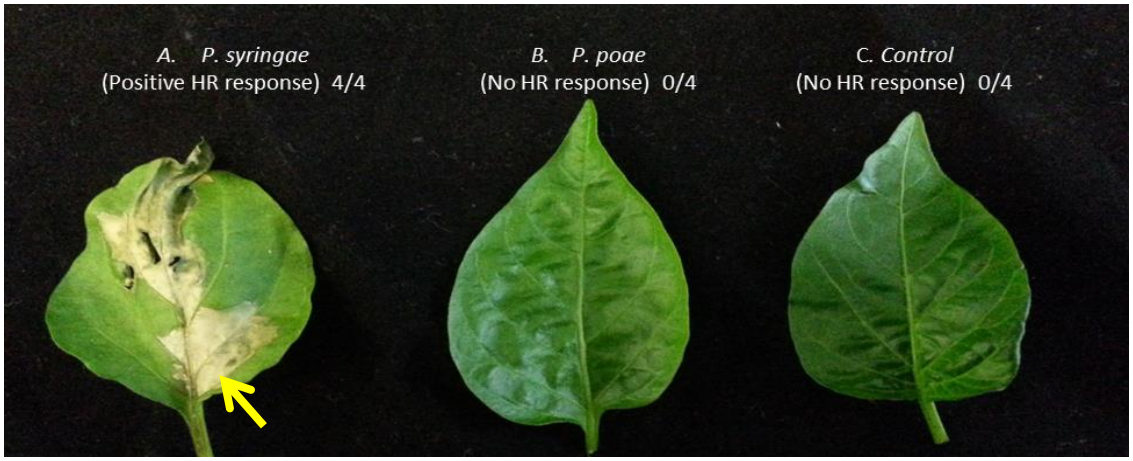


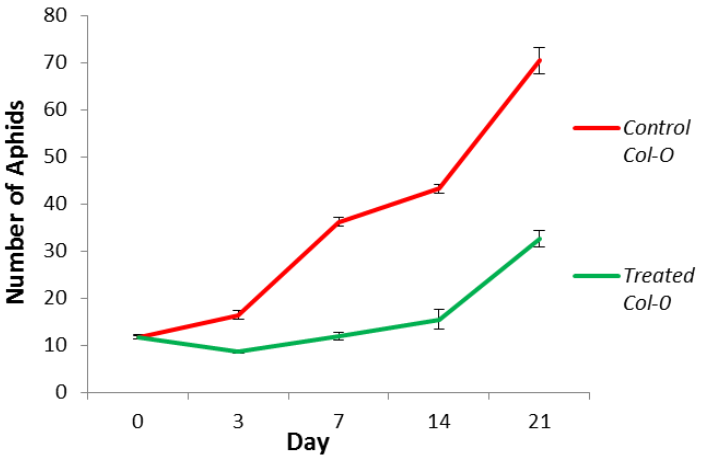
Figure 4.3: Assessment of Hypersensitive response (HR) in peppers after foliar spray of different bacteria at 3 day post inoculation (dpi). Different bacteria strains 10^{7} CFU mL^{-1} sprayed on pepper plants. At day 3, Yellow arrow indicates leaf showing HR. A. *P. syringae pv tomato DC3000* - Positive HR response, B. *P. Poae* - No HR response and C. Control (water) No HR response. The numbers of infected individual plants of the four plants per treatment are indicated.

4.2.2 Effect on the aphid population after *P. poae* spray

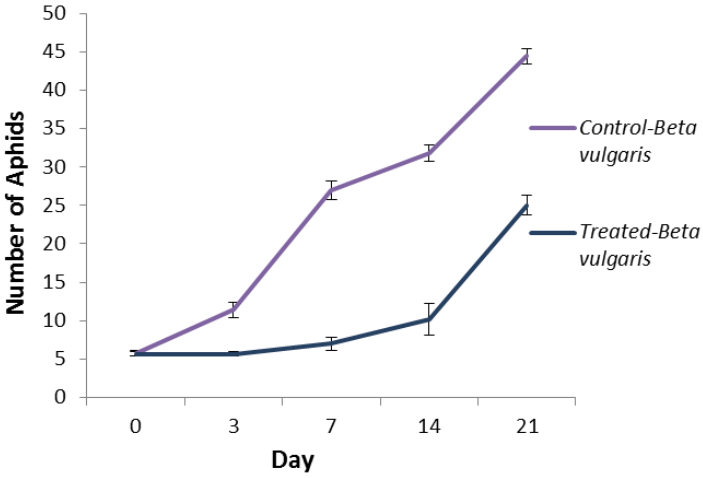
As *P. poae* was observed to survive in the plant environment for three weeks for all of the plant conditions tested, I next decided to determine whether *P. poae* inoculated plants can control aphid infestation efficiently.

The same numbers of aphids were introduced on the bacteria-inoculated and non-inoculated (water control) plants species on the same day of bacterial inoculation (Day-0). The aphid counts, which represented both nymphs and adults, were recorded on control and treated plants during the course of three weeks. It was observed that all control plants had aphid populations were continually growing and maintaining threshold level of infestation. However, a significant decline in the number of nymphs on bacteria-inoculated tested plants was seen, which resulted in a final reduction of aphid populations in *Arabidopsis* (Col-0), pepper & sugar beet by 57 %, 68 %, 69 % respectively comparing with control aphid populations (Figure 4.4). Control & treated aphid counts for each of the plant species from all time points were significantly different at p value < 0.05.

A - *Arabidopsis thaliana*



B - *Beta vulgaris*



C - *Capsicum annum*

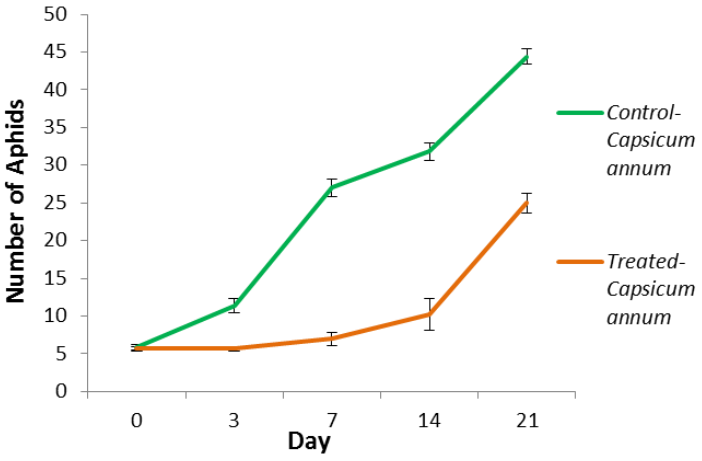


Figure 4.4: Effect on aphid leaf populations after *P. poae* spray on different plants. Aphid populations were recorded from non-inoculated (control) & inoculated (treated) plants A) *Arabidopsis thaliana* (Col-0 ecotype) B) *Beta vulgaris* C) *Capsicum annum* over period of 21 days. The data presented are the mean and standard error of six biological replicates.

Additionally, the assessment of *P. poae* efficacy was done at different elapsed time intervals after spraying, by scoring surviving pests. In this experiment, the bacterial spray methodology was used, but the introduction of 50 aphids on control and treated plants was done at 0, 3, 7, 14 and 21 days. The aphid counts were recorded to examine the percentage control at different time intervals in relation to the aphid infestation level of control plants (Table 4.1). *P. poae* provided excellent control of aphids with a ranging 61-88 % efficacy rate after foliar application at all assessment intervals. The 88 % efficacy control rate observed at 7 days after application was significantly higher than other time intervals (*$p < 0.01$).

Table 4.1: *P. poae* efficacy to control aphids on peppers.

Time	Reduction in Aphid populations (%)	Standard error
Day 3	61.43	4.96
Day 7	88.30	2.66
Day 14	76.49	4.26
Day 21	82.93	2.55

The reduction in aphid populations evaluated on peppers on different days after application (DAA) of *P. poae*. The data presented are the mean and standard error of five biological replicates.

4.2.3 Aphid behavioural assay

The *in vitro* killing effect of the bacteria has already been established, but the *in planta* effect needs to be investigated further. In addition to bacterial toxicity, the decline in number of aphid populations on bacteria-treated plants may indicate the changes in behavioural cues of aphid that could include feeding behaviour, host plant choice and other olfactory cues. To understand the interaction of the aphid on the bacteria-treated plants, a preliminary choice and no choice experiment was carried out. Ten 3 weeks old pepper plants (each with 8-9 leaves per plants) were placed into an aphid tent that measured $60cm^3$. Bacteria were sprayed on five pepper plants and while another five plants were sprayed with sterile water. 40-50 aphids were removed from infested plants and kept in parafilm lined Perspex tubes for starving for at least 3 h. Finally, they were placed in the middle of the tent floor and allowed to migrate to any of the ten plants contained in the tent. Similarly, five pepper plants sprayed with bacteria and five sprayed with sterile water were placed in two separate tents and 25-30 starved aphids placed in the each tent. In all cases, numbers of aphids on each plant were monitored 1, 2, 3, 7, 14 and 21 days after the aphids were introduced.

When given no choice, aphids colonised both *P. poae* and water spray plants in separate tents, although the aphid populations on bacteria-treated plants were significantly lower than control plants (*$p < 0.05$) (Figure 4.5). However, when aphids had the choice of inoculated or

non-inoculated plants they selected to colonise the non-inoculated plant before the inoculated plant. The number of aphids were significantly higher on control water spray plants than bacteria spray plants (*p < 0.05) (Figure 4.6). These findings suggested an altered aphid olfactory response to volatiles from bacteria-treated plants. To investigate this altered behaviour, an olfactory assay was conducted on winged aphids by collection of volatiles from plants alone, bacteria alone and bacteria-treated plants. In this approach, I observed aphid behaviour in terms of time spent in treated and control arms of an olfactometer and the data were analysed statistically to score attractant or repellent behaviour. In this study, an olfactometer was programmed with Olga software, which is able to detect one treated arm time and compared with other solvent control. Hence, separate olfactometer analysis was performed through use of volatiles collected from different treatments. After a 2-day volatile collection from *P. poae* streaked LA plates, the olfactometer assay showed no significant difference in time spent in control and treated arm (*p >0.05) (Figure 4.7).

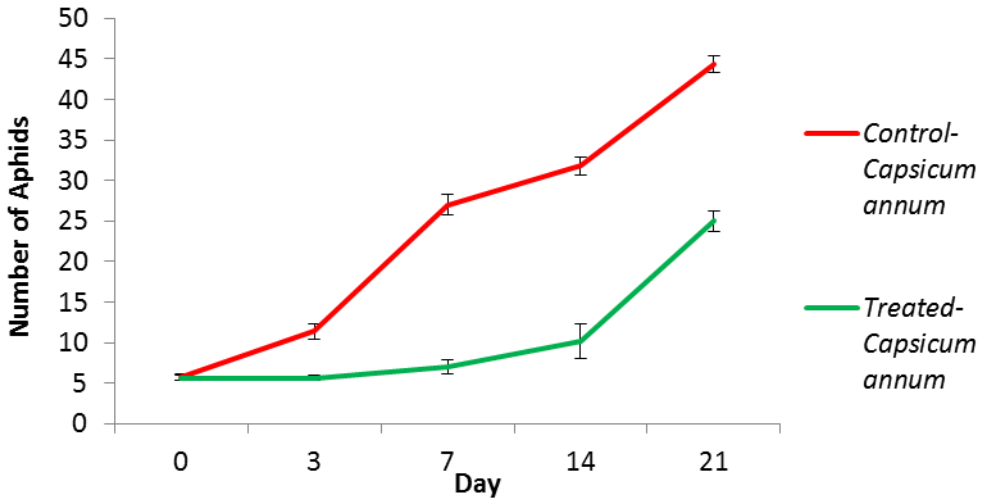


Figure 4.5: Enumeration of aphid populations on peppers in no choice experiment. Aphid populations recorded from non-inoculated (control) & inoculated (treated) *Capsicum annum* plants over period of 21 days in separate tents. The data presented are the mean and standard error of five biological replicates.

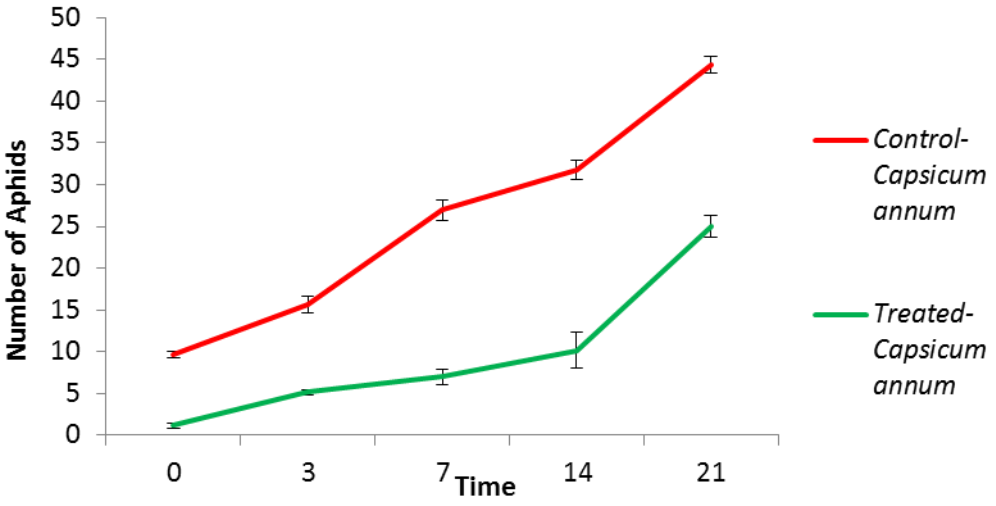


Figure 4.6: Enumeration of aphid populations on peppers in choice experiment. Aphid populations recorded from non-inoculated (control) & inoculated (treated) *Capsicum annum* plants over period of 21 days in same tents. The data presented are the mean and standard error of five biological replicates.

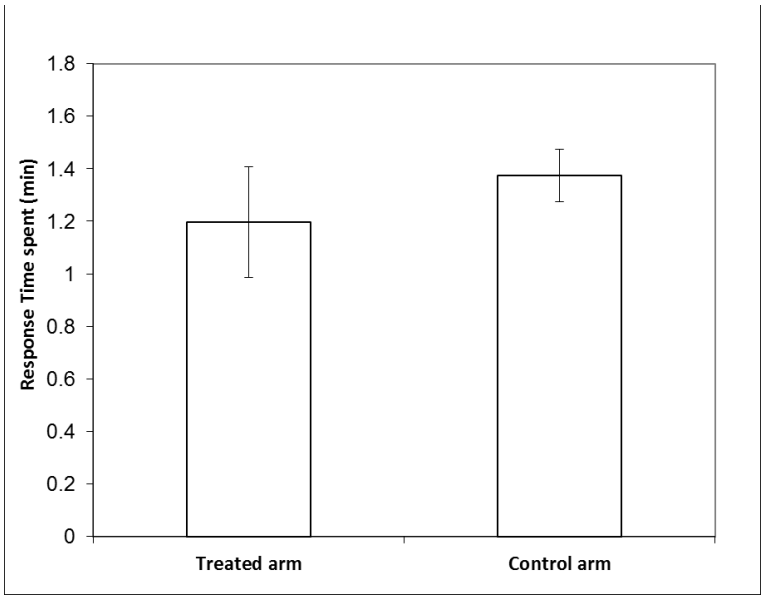


Figure 4.7: Olfactometer assay on *P. poae* volatiles. Time spent by winged aphid recorded in each arm of olfactometer. The treated arm represents *P. poae* volatiles in diethyl ether solvent whereas the control arm was assigned to standard diethyl ether solution. The data presented are the mean and standard error of ten biological replicates.

In a second olfactometer assay, volatiles were extracted from *P. poae* inoculated (treated) and non-inoculated (control) plants for 48 h. It was observed that aphids spent significantly less time in the arm containing the treated volatiles as compared to control volatiles (*$p < 0.05$) (Figure 4.8 b). This altered aphid behaviour represented repellency or deterrence towards bacteria-treated plant volatiles.

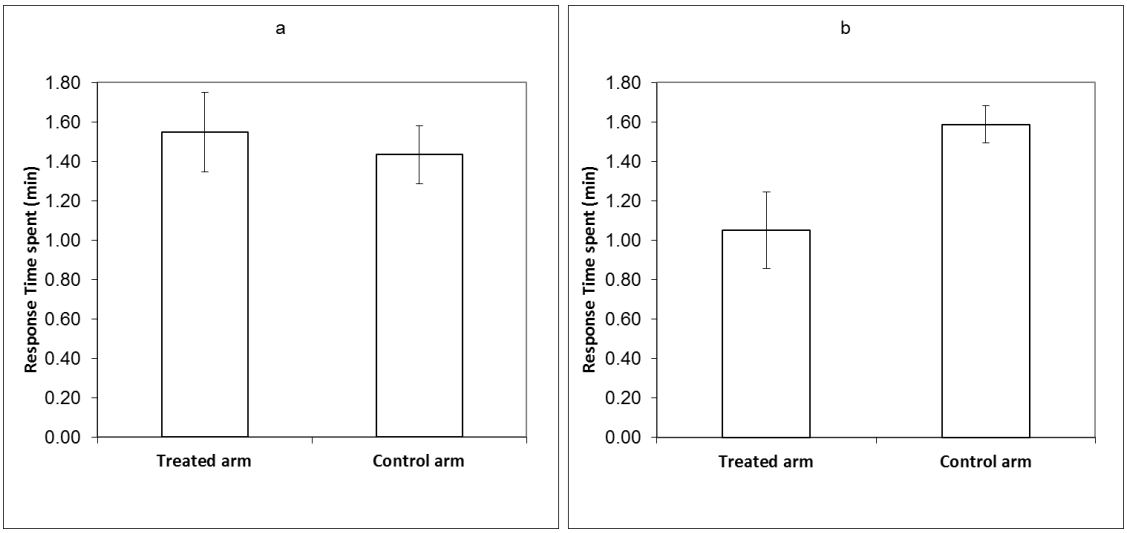


Figure 4.8: Olfactometer assay on *P. poae* – pepper volatiles. Time spent by winged aphid recorded in each arm of olfactometer when exposed to **a)** Volatiles from pepper plants inoculated with water **b)** Volatiles from pepper plants inoculated with *P. poae* (10^{7} CFU mL^{-1}). The treated arm represents *P. poae* volatiles in diethyl ether solvent whereas the control arm was assigned to standard diethyl ether solution. The data presented are the mean and standard error of ten biological replicates.

From the above experiments on repellency behaviour and choice experiment, the data strongly suggested that *P. poae* treatment on plants affects the rate of aphid colonisation.

4.3 Discussion

The fluorescent Pseudomonads are highly adaptive and can use a wide variety of compounds as an energy source, and as a result, they can colonize different environmental niches (Silby *et al.,* 2011; Wu *et al.,* 2011). They are able to survive in a wide range of environments from oil-spilled sea water to soil, plant surfaces and insect guts (Hirano & Upper, 2000; Weller *et al.,* 2002; Vodovar *et al.,* 2005; Viggor *et al.,* 2013). They are known to enhance plant growth promotion, induced systemic resistance and reduce severity of fungal diseases (Hoffland, 1996; Wei *et al.,* 1996). Notably, strains of *P. fluorescens* have been shown insecticidal activity toward agricultural pest insects such as aphids, phytophagous ladybird beetles and termites (Hashimoto, 2002; Otsu *et al.,* 2004; Devi & Kothamasi, 2009). Successful application of *Pseudomonas* by seed treatment and foliar spray well established in control of *Pythium*, Rhizoctonia, and *Fusarium* root diseases of vegetables and ornamentals in greenhouses (Fravel, 2005). This study demonstrated that foliar spray of *P. protegens* strain CHA0 and *P. chlororaphis* strain PCL1391 efficiently killed larvae of many agriculturally important lepidopteran pest insects, notably African cotton leafworm *Spodoptera littoralis*, the tobacco budworm *Heliothis virescens*, and the diamondback moth *Plutella xylostella* (Ruffner, 2013b).

In the current study, the newly isolated *P. poae* displayed potent oral insecticidal activity in feeding assays with artificial diet and leaves treated with the bacteria. Foliar spray of *P. poae* successfully reduced aphid populations by an average rate of 55 % on three different plant species (*Arabidopsis*, sugar beet, and peppers) over a period of three weeks (Figure 4.3). Moreover, the average population of log 20-25 bacterial CFU cm^{-2} remained stable for 21 days without any hypersensitive response on plants that strongly supported the ability of *P. poae* to colonise different plants, each of which likely have differential carbon and nitrogen sources (Figure 4.2) (Ganeshan & Manoj Kumar, 2005). All these results are supported by research which successfully demonstrated foliar spray of *Pseudomonas* to control powdery mildew of pea and other plant pathogens (Bahadur *et al*., 2007). However, in this study, a seed treatment method did not work effectively due to low moisture content, high pH in the soil, and the presence of abrasive shear forces, which causes cell lysis. Other major seed inoculation parameters (McQuilken *et al.,* 1998) such as osmoprotectants, damp seed incubation (moisture content) and priming method were not effectively regulated.

Hence, it is concluded that the improved talc- and kaolin-based seed bio formulation method which described standardized seed inoculation parameters to formulate *Pseudomonas chlororaphis* 63-28 *strain* for disease control could be tested in future studies (Correa *et al.,* 2015).

The current study showed that *P. poae* efficacy to control aphid populations was highest at day-7 with an 86 % percentage control while testing different time intervals (Table 4.1). These results confirmed similar findings of highest efficacy rate to control aphids and whitefly at day-7 by use of butenolide insecticide (Ralf Nauen *et al.,* 2015). Another study evaluating the efficacy of insecticide in field trials, found that flupyradifurone showed excellent efficacy against other sucking pests such as *Dysaphis plantaginea* and *Aphis pomi* in apples; *A. gossypii*, *M. persicae*, *Trialeurodes vaporariorum* and *Bemisia tabaci* in vegetables; and *Empoasca flavescens* and *Scaphoideus titanus* in grapes, with different application methods, including foliar, soil, seed treatment and drip irrigation (Roffeni *et.al.,* 2014). Furthermore, flupyradifurone provided the highest level of control against lettuce aphids at 6–10 days after application, i.e. 96 % efficacy.

Additionally, several Pseudomonads are plant growth-promoting rhizobacteria (PGPR), that increase plant growth and yield under greenhouse and field conditions, often eliciting induced resistance referred to as Induced systemic resistance. Several research studies have revealed that rhizobacteria, including *Pseudomonas* and *Bacillus;* emit volatile organic compounds, which can modulate plant defences to reduce fungal severity(Scala *et al.,* 2013; Abdul *et al.,* 2017) . Additionally, these VOCs also trigger expression of genes involved in plant green leaf volatile signalling pathway to attract natural enemies of pests, an indirect defence strategy that protects plants from herbivores (Vander Ent *et al.,* 2009; Barco *et al.,* 2010). The plant beneficial bacterium *Pseudomonas fluorescens* WCS417r was known to modulate JA or ET signalling, resulting in induced expression of defence-associated genes, which enhanced the level of resistance to the herbivores(Wees *et al.,* 2008; Vander Ent *et al.,* 2009). In plants, synthesis of VOCs such as Green Leaf Volatiles (GLVs) and terpenoids are regulated by JA and shikimate pathway and the emission of volatiles such as methyl salicylate (MeSA) are regulated by SA (Dicke, 2002; Van Poecke and Dicke, 2002; Maffei *et al.,* 2011). The JA plant hormone is major player of both ISR and plant defences against herbivorous insects, therefore plant beneficial bacteria are expected to affect plant–insect interactions. Furthermore, the role of visual (use of reflective mulches & yellow sticky trap) and olfactory cues of aphids to attractant or repellents of different plant volatiles have been well studied in *Aphis fabae* by various olfactometer assays (Nottingham & Hardie, 1993; Hardie *et al.,* 1994).

Similar aphid behavioural investigations were carried out in this current study through olfactometer assays. The *P. poae*-only assay showed no significant difference in control and treated arm, which indicated that no volatile organic compound released from *P. poae* can affect performance of aphid behaviour negatively or positively (Figure 4.7). To examine the role of plant-bacteria interactions another olfactometer assay was carried out on volatiles

collected from non-inoculated and *P. poae*-inoculated pepper plants. Olfactometer bioassays using volatile samples from water sprayed peppers showed no effect on the aphid behaviour (Figure 4.8 a). However, *P. poae* induced pepper volatiles can affect the behaviour of aphids by making plant less attractive to them that led to reduction in their infestation (Figure 4.8 b).

Moreover, the choice experiment depicted that aphids are more likely to colonise the non-inoculated peppers, which led to a decline in aphid populations on inoculated pepper plants. Such repellency behaviour of *M. persicae* against *P. poae* treated pepper volatiles suggested that in addition to toxicity towards aphids, this bacterium might have an important role in modifying the regime of green leaf volatiles by interfering in phytohormonal signalling pathway.

Although, I had not initially envisaged discovery of *P. poae*-treated pepper volatiles, it would be useful in the future to carry out a chemical analysis of these volatiles coupled by gas chromatography –electroantennography (GC-EAG), to help explore how aphid olfactory sensilla respond to bacteria treated volatiles. This analytical procedure allows rapid identification of compounds in complex mixtures that stimulate the olfactory sensilla of an insect. The identification and characterization of volatile compound might be helpful in future pest management strategies as it could be exploited as an adjuvant with microbial pesticides to repel the aphids. Additionally in this study, the four arm olfactometer design had only one treated arm which resulted in separate analysis of different volatiles. Hence, future olfactometer studies with advanced six arms can allow the investigation of volatiles collected from water and bacteria-treated spray together in a single observation. This logistic approach would provide the amount of time spent by aphids in two different treated arms as compared to control solvent and help to investigate how volatiles affect aphid behaviour.

In summary, the current work represents a new biological control candidate "*Pseudomonas poae*" to control aphid infestation. Considering the application method, the foliar application method showed excellent efficiency and provided three week longevity of bacterial colonisation along with an average 55 % control of aphids on tested plants. For the commercial use, the effect of polymeric additives, adjuvants, and surfactants on survival, stability, biocontrol and plant growth promoting ability of *P. poae* should be performed in greenhouses or field trials to study its long term effects. The application of *P. poae* on plants appears to have released volatiles, probably as a response of systemic resistance which might affect feeding behaviour of aphid. In future, these behavioural investigations will help to understand how to synchronize the release of aphids to recognize suitable phenological stages of the bacteria-treated plant by its specific odour. Besides these, the recent molecular characterization of the *P. poae* genome revealed a number of potential insecticidal toxin and

other genes putatively involved in secondary metabolite production (e.g. siderophores, phenolics and antibiotics). Therefore, further investigation on the above candidate genes will be helpful to understand the virulence towards aphids.

5 An examination of alterations in host gene expression after infection of *Pseudomonas poae* to aphids

5.1 Introduction

Aphids are often challenged simultaneously by multiple environmental stresses in their natural habitat. There are numerous forms of stress, including extreme temperature, drought, pathogens and parasites. Examples of the latter are parasitoid wasps, which consume their hosts as they grow inside, and viral, bacterial and fungal pathogens. Both parasitoid wasps and fungal pathogens are responsible for controlling natural aphid population and are potential agents for biocontrol of these phytophagous pests (Hufbauer, 2002; Snyder & Ives, 2003).

The invasion of pathogens and parasites into an insect is defended by an innate immune system, a strong universal defence mechanism shared by both vertebrates and invertebrates (Gillespie *et al.,* 1997). The first line of defence includes physical barriers such as the protective cuticle and gut pH, which prevent entry of many pathogens. Unlike vertebrates, insects do not have adaptive immunity or antigen based immune response but cellular responses involve phagocytosis and encapsulation by the circulating haemocytes. The humoral response refers to the process of melanisation and the production of immune effector molecules, which are mainly produced in the fat body. Anti-bacterial immunity depends on two principal signalling pathways, Toll and **Im**mune **D**eficiency (IMD), which are conserved across various insect species indicating their central importance throughout arthropod evolution (Ferrandon *et al.,* 2007; Lemaitre & Hoffmann, 2007).

To date, an insect's immune system has been thought to be restricted to the innate response rather than specific based immunity (for example, the antigen-based immune response of humans). There is, however, increasing evidence for the ability of insects to mount specific immune responses (Schulenburg *et al.,* 2007). The recent sequencing of the pea aphid provided novel insights into the immune and stress gene repertoire of aphids, and provided evidence of a reduced immune response as compared to others insects. The gene underlying immune responses in other insects (e.g. Toll and **Ja**nus **K**inase/**S**ignal **T**ransducer and **A**ctivator of **T**ranscription (JAK/STAT) pathway genes) were present in the aphid genome but major bacteria recognition genes were missing from the immune repertoire. For example, typical insect antibacterial peptides (including defensins, attacins, and cecropins) and essential genes involved in the IMD pathway (including peptidoglycan recognition proteins (PGRPs) and the central IMD protein) are absent. Both transcriptomic and proteomic analysis revealed few up-regulated products in microbe exposed pea aphids compared with unchallenged aphids

(Altincicek *et al.,* 2011). Furthermore, immunological analysis has demonstrated that *A. pisum* displays only weak lysozyme-like activity, haemolymph coagulation, and phenol oxidase activation reactions (Laughton *et al.,* 2011).

Altincicek *et al.* (2011) suggested the two major reasons for the reduced immune response in pea aphid; 1) high rate of reproduction investment and 2) symbiont mediated host response (Gerardo *et al.,* 2010). To test this hypothesis, further characterization of global aphid stress and immune response under different conditions is required. Additionally, aphid relatives with different habitats (for example, those not associated with secondary symbionts, or those that live in soil or other microbe rich habitats) may be particularly helpful to fully understand the stress and immune response of aphids.

Finally, an overview of the tissue-specific gene expression profiles in response to infection and a platform for further exploring the molecular basis of the host antimicrobial response would strengthen our understanding of immune and other defence mechanisms, and could lead to the exploitation of microbes in managing aphid infestations.

In this study, a range of plant-associated bacteria were discovered, mainly *Pseudomonas*, but also *Pantoea*, *Acinetobacter* and *Paenibacillus*, that cause death of aphids after ingestion (Figure 3.1). This is an exciting discovery because it suggests plants harbour a range of aphicidal bacterial communities that may act as natural antagonists for aphid colonisation. Importantly, these bacteria hold promise for development as natural biocontrol agents to control aphid colonisation and infestation of crop plants.

Some previous work done on insect-bacterial interactions has shown that bacteria can kill insects through the production of insecticidal toxins or by occlusion of the insect gut and death by starvation. Some of our initial evidence suggests that some of our bacteria secrete a toxic compound, while live bacteria are required for the killing effect. This suggests a diversity of killing effects are in operation by different bacteria.

I identified *Pseudomonas poae* as the most potent pathogenic bacterium that kills aphids in 48 h (Figure 3.3 A). Genome analysis data of *P. poae* revealed five different insecticidal toxins, stress response genes and other pathogenicity related effectors genes which may confer toxicity towards aphids (Chapter-6 in this study). A logical next step would be to explore the involvement of these genes in pathogenicity by analysing the molecular changes occurring in the bacterium and the aphid during infection. From this perspective, a comprehensive large-scale transcriptional study was required to extend this work and characterise the full complementation of the aphid response to bacterial ingestion. Hence, RNA sequencing was

employed to analyse the molecular changes occurring in the bacterium and the aphid during an infection.

RNA-Seq or deep sequencing of cDNA libraries by next generation sequencing is a sensitive way of profiling both prokaryotic and eukaryotic gene expression from bacteria-infected cells. RNA-Seq is annotation independent, allowing novel transcript discovery without being reliant on array design or pre-existing annotation. Unlike tag sequencing, RNA-Seq can distinguish different mRNA isoforms and non-cytoplasmic RNA, and can identify splice junctions and transcript boundaries. Despite these advantages, dual host-pathogen RNA-Seq is technically challenging as total RNA extracted from infected cells are a mixture of host and bacteria RNA. Furthermore, bacterial RNA is typically a very minor fraction of infected cells, even under optimized *in vitro* conditions, and especially in early infection periods where bacterial numbers can be low. To get maximum coverage of both host-pathogen transcripts, mRNA enrichment steps are required. In heterogeneous mixture of total RNA, 98 % ribosomal RNA can be removed by ribo depletion and the remaining 1-2 % coding mRNA could be enriched for subsequent analysis. This allows optimal number of bacteria transcripts to be obtained from mixed infected RNA.

In this chapter I performed aphid mortality assays and isolated three different RNA samples across two conditions (control & treated) (Fig. 5.1):

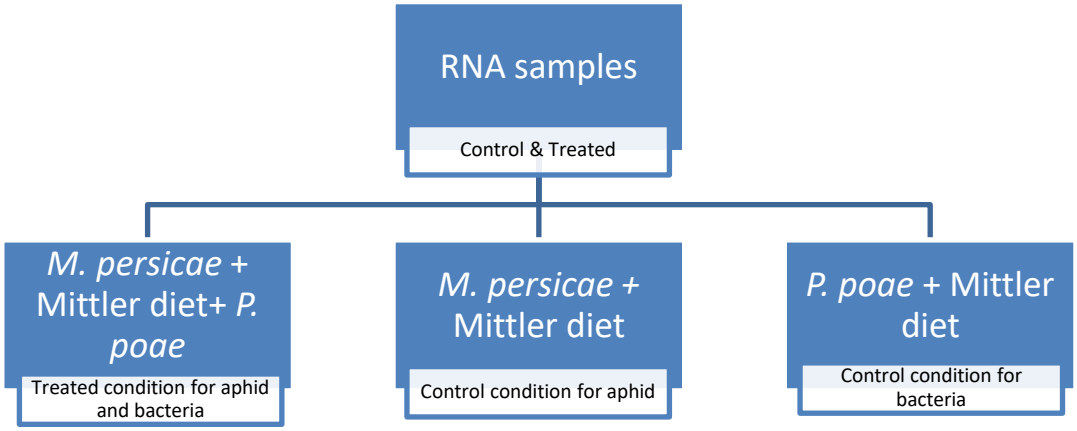


Figure 5.1: RNA samples across control and treated conditions. In the aphid mortality assay, two conditions were employed; both aphid and bacteria in Mittler diet titled "control" condition and aphid fed on Mittler diet with bacteria cells titled 'treated' condition.

My aim was to determine:

- The changes in the bacterium during aphid infection (treated) & compare with bacterial gene expression in Mittler diet (control).
- The changes in aphid gene expression between bacteria infected (treated) & non-infected (control) aphids.

5.2 Methods

5.2.1 RNA Extractions from Bacterial Cultures

To extract RNA from *Pseudomonas poae*, a single bacterial colony was inoculated in Mittler diet at 18 °C. The RNA Protect Bacteria Reagent (QIAGEN, Limburg, Netherlands) was used to avoid bacterial transcripts degradation, according to the manufacturer's instructions. Bacterial culture was suspended in twice volume of reagent, mixed for ten seconds and left for ten minutes at room temperature. Bacterial cell was pelleted by centrifugation at 1,2000 g for ten minutes. The supernatant was discarded and the pellet either subjected to RNA isolation step or stored for later use at -80 °C. Total RNA was then extracted from the samples using the RNeasy Mini kit protocol (QIAGEN, Limburg, Netherlands), with the addition of the optional DNase I digestion stage for fifteen minutes (QIAGEN, Limburg, Netherlands). The second gDNA removal was performed through use of DNase treatment in the TURBO DNA-free protocol (Ambion, Life Technologies, Carlsbad, USA). Total RNA concentration was estimated by NanoDrop (Wilmington, USA) spectrophotometer and total RNA integrity was visualised in a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). Additionally, RNA samples were also visualized on 1 % nuclease-free agarose gel loaded with RNase-free 2X RNA loading dye (New England Biolabs, Ipswich, USA).

5.2.2 RNA Extractions from Aphids

The UK sensitive aphid clone 4106A was used for subsequent aphid transcript analysis. Total RNA of control and infected aphids was prepared using the RNeasy Mini kit (Qiagen). To extract total RNA from aphids, control and bacteria inoculated aphid sachets (described in section 2.5 Chapter 2) were prepared in four replicates.

Some modification of the aphid mortality assay was done to extract RNA from infected aphids. Four biological different bacteria replicate cultures were used to infected aphids in four different sachets each consisting of 10 aphids. After 38 h feeding, treated aphids from each bacterial replicate were pooled (4 x 10 aphids) and crushed in lysis buffer (700 µL) in a micro

centrifuge tube, with a sterilized pestle. Control aphids were pooled (4 x 10 aphids) from non-inoculated aphid sachets and ground in lysis buffer in the same way.

Total RNA was then extracted from the samples using the RNeasy Mini kit protocol (QIAGEN, Limburg, Netherlands), with the addition of the optional DNase I digestion stage for fifteen minutes (QIAGEN, Limburg, Netherlands). The second gDNA removal was performed through use of DNase treatment in the TURBO DNA-free protocol (Ambion, Life Technologies, Carlsbad, USA). Total RNA concentration was estimated by NanoDrop (Wilmington, USA) spectrophotometer and total RNA integrity was visualised in a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). Additionally, RNA samples were also visualized on 1 % nuclease-free agarose gel loaded with RNase-free 2X RNA loading dye (New England Biolabs, Ipswich, USA).

5.2.3 Bacteria total RNA enrichment from infected aphids

The majority of bacterial infected aphid total RNA comprises aphid RNA with RNA of the bacterial pathogen the minor fraction.

An improved methodology was employed to enrich the bacterial RNA component from total RNA of infected aphids, where oligo mix was used to remove >90 % of the eukaryotic 18S and 28S rRNAs, and polyadenylated mRNAs form these mixtures. The 25 µg (maximum amount) of total RNA from infected aphids was used as input RNA for enriching bacterial RNA by using MICROBEnrich™ Kit (Life Technologies, Carlsbad, USA), following the manufacturer's protocol. The ethanol precipitation method was used for total RNA purification. The concentration and quality of total RNA obtained was checked as detailed above.

5.2.4 Ribosomal RNA (r-RNA) depletion

Ribosomal RNA (rRNA) constitutes the predominant fraction of the transcriptome. To avoid wasting sequencing effort on a few superabundant molecules, rRNA needs to be removed prior to library preparation. Total RNA of aphid (control & treated), control Bacteria & enriched bacteria RNA samples were enriched for mRNA by using the Ribo-Zero rRNA removal kit (Illumina, San Diego, U.S.A), following the manufacturer's protocol. The rRNA depleted RNA quality was assessed by Agilent 2100 Bioanalyzer, through use of the Agilent RNA6000 Pico Chip according to the manufacturer's instructions.

5.3 Preparation of RNA for qPCR analysis

5.3.1 Removal of gDNA contamination

After quality assessment of RNA samples on a NanoDrop spectrophotometer and quality check on the Bioanalyzer, a quantitative polymerase chain reaction (see QPCR reaction setup and cycling conditions) was performed on all samples to assess the presence of genomic DNA (gDNA) using housekeeping genes. Majorly bacteria samples showed gDNA contamination which required another DNA removal, using the routine DNase treatment in the TURBO DNA-free protocol (Ambion, Life Technologies, Carlsbad, USA). Whereas, no gDNA contamination have been reported in aphid RNA samples. The confirmation of gDNA removal was performed by QPCR on the samples: a negative sample which showed PCR amplification after 34 cycle and assumed that there was no gDNA contamination in the RNA (along with positive, gDNA and negative no-template controls).

5.3.2 cDNA synthesis

A total of 4 µg of total RNA was converted into cDNA samples using Superscript II reagents and the protocol for using random primers as described by the manufacturer (Life Technologies, Carlsbad, USA), but including the 11mer primers, described above (Fislage *et al.,* 1997).

5.4 qPCR analysis

5.4.1 qPCR Setup, Cycling Conditions and Analysis

All qPCR reactions were set up using SYBR® Green JumpStart™ Taq Ready-mix (here in referred to as JumpStart Taq ReadyMix) (Sigma-Aldrich Company Ltd, Dorset, U.K.) as follows: 10 µL 2 x JumpStart Taq ReadyMix SYBR; 0.5 µL 10 µM forward primer; 0.5 µL 10 µM reverse primer; 4 µL cDNA at 1/8 dilution; DNase-, RNase-free molecular biology grade water to 20 µL, in 72-wells. Reactions were carried out using a Corbett Rotor-Gene 6000 machine (QIAGEN, Limburg, Netherlands) with the following cycling conditions: initial denaturation at 95 °C for 10 minutes; 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C for 15 s and extension at 72 °C for 15 s. A final melt-curve step was included post-PCR (ramping from 72 °C–95 °C by 1 °C every 5 seconds) to confirm the absence of any non-specific amplification. Data was analysed by averaging three technical and four biological replicates and applying the formula $2-\Delta\Delta CT$, with the data being normalised to the calibrator (control) sample and to a selected reference gene to obtain the fold-change in product levels.

5.4.2 Optimisation of Primer Efficiency

The efficiency of each qPCR primer to generate PCR product was conducted by a standard curve qPCR reaction, with serial dilutions from a RNA sample and no template control. To test precision and working range of qPCR primer, five 10-fold dilutions of cDNA were prepared in nuclease free water, starting at a concentration of 20 ng μL^{-1} cDNA. The cDNA was prepared from the calibrator sample of aphid & bacteria in Mittler die at 18 °C for 38 h (as described for the treatment control condition for aphid bioassay).

The qPCR software prepared a standard curve by plotting the log of RNA input level against the Ct value for each primer set and calculated the efficiency. Finally, the amplification factor was calculated by the slope of the line regression by using the formula (10^ (-1/slope value)).

All qPCR primers detailed in Table 2.5 (Chapter 2) were tested, however only primers with an efficiency of 95-105 % were used for further qPCR (Table 5.1).

Table 5.1: Primer Efficiency test for Quantitative PCR.				
Target Gene	**qPCR Primers**	**R^2**	**Efficiency**	**Amplification factor**
Venom protease	Mp_Vp_F1/R1	0.98	1.01	2.01
Cathepsin B-N	Mp_cathepsin_F1/R1	0.99	1.01	2.01
Noggin	Mp_Ng_F1/R1	0.99	1.12	2.12
Noggin	Mp_Ng_F3/R3	0.99	1.16	2.16
Larval cuticle	Mp_Cuticle_F3/R3	0.98	1.05	2.05
Alpha-tocopherol	Mp_Toco_F2/R2	0.99	1.03	2.03
Cytochrome P450 6a13	Mp_cycP450_F1/R1	0.97	1.09	2.09
Carotenoid desaturase, partial	Mp_CAT_F1/R1	0.98	1.01	2.01
Carotenoid desaturase, partial	Mp_CAT_F2/R2	0.98	0.95	1.95
Gamma-glutamyltranspeptidase	Mp_ggt_F1/R1	0.98	1.09	2.1
Olfactory receptor	Mp_OF_F1/R1	0.97	1.1	2.1
Olfactory receptor	Mp_OF_F2/R2	0.99	1.01	2.01
Major facilitator superfamily domain-containing 6-like	Mp_mfs_F2/R2	0.99	1.01	2.01
Major facilitator superfamily domain-containing 6-like	Mp_mfs_F4/R4	0.99	1.05	2.05
Facilitated trehalose transporter Tret1	Mp_Tre_F2/R2	0.99	1.01	2.01
Facilitated trehalose transporter Tret1	Mp_Tre_F4/R4	0.99	1.08	2.09
legumain	Mp_lg_F2/R2	0.98	1.1	2.1
Lycopene	Mp_lyco_F1/R1	0.98	1.14	2.14
Actin	MpActF1/R1	0.99	0.98	1.98
Sodium Channel	Mp_Aph1R/	0.97	1.06	2.06
AprX-Serine protease	Poae_aprA_F1/R1	0.99	0.97	1.97
AprX-Serine protease	Poae_aprA_F3/R3	0.99	0.97	1.97
PvdD-NRPS	Poae_pvdD_F1/R1	0.99	0.94	1.96
PvdF-synthetase (Pyoverdine biosynthesis)	Poae_pvdF_F1/R1	0.99	0.97	1.97
EfeoB1 (peroxidase)	Poae_EfeB_F1/R1	0.99	0.94	1.96
EfeoB1 (peroxidase)	Poae_EfeB_F/R	0.99	0.92	1.92
Thymine DNA-glycosylase	Poae_mug_F1/R1	0.99	0.93	1.93
AHYP -Alkyl hydroperoxide reductase	Poae_AHYP_F1/R1	0.99	0.93	1.93
RND efflux membrane fusion protein	Poae_RND_F2/R2	0.98	0.97	1.97
Fimbriae usher protein StfC	Poae_fimbriae_F1/R1	0.99	0.97	1.97
Arginine deaminase	Poae_arcA_F2/R2	0.99	0.93	1.95
Hypothetical protein-toxin	Poae_Hyp_F2/R2	0.99	0.92	1.92
Hypothetical protein-toxin	Poae_Hyp_F3/R3	0.97	1.08	2.08
Haem oxygenase	poae_HOX_F2/R2	0.97	1.05	2.05
TcaA toxin	Poae_TcaA_F/R	0.99	0.93	1.95
TcaA toxin	Poae_TcaA_F2/R2	0.99	0.97	1.97
TccC toxin	Poae_TccC_F2/R2	0.97	0.92	1.94
RpoD	Poae_rpoDF1/R1	0.98	0.93	1.93
RpoS	Poae_rpoSF1/R1	0.97	0.92	1.94
Primers were tested for their optimum efficiency on the five diluted control cDNA samples. The slope of linear Cycle threshold CT values and cDNA concentration curve was used to calculate efficiency E = 10(-1/slope) – 1.				

5.5 RNA sequencing design

To determine the coverage of the RNA Seq experiment is difficult because different transcripts are expressed at different levels, which mean high coverage of highly expressed genes and low coverage of low expressed genes. Additionally, other factors such as complexity in the transcriptome, alternate expression, and 3'-associated biases also make it difficult to calculate coverage elements. Hence, the total number of mapped reads is the best characterized metric to analyse RNA Seq coverage. On the basis of genome size and sample, minimum reads for small genomes (bacteria/fungi) are 5 million, intermediate genomes (insects, *Caenorhabditis elegans*) are 10 million and large genomes (Human / Mouse) are 15-25 million (Liu *et al.,* 2014).

Therefore, the above recommended sequence coverage of RNA Seq for differential expression profiling was followed in the current study. The distribution of aphid and bacteria RNA samples across the lanes was performed in this way to gain a minimum 10-15 million reads for the aphid and 5-10 million reads for the bacteria to get maximum sequence coverage.

In the first RNA-Seq, *Myzus persicae* 4106A aphid clone was fed with 10^2 CFU mL^{-1} infection dose for 48 h. RNA isolation was carried out from control and treated samples of both aphid and bacteria in three replicates which was further subjected to ribodepletion (section 5.2). A total of nine RNA samples were sent to Centre for Genomic Research - University of Liverpool for paired-end sequencing (2 x 100 bp). After c-DNA synthesis (Illumina TruSeq RNA libraries), nine indexed libraries multiplexed into two lanes of the Illumina HiSeq platform, generating data in excess of 120M clusters per lane. To increase sequence depth of treated bacterial transcripts (bacteria from infected aphids), three ribodepleted bacteria treated aphid samples were allowed to run on single lane and a second lane was used for both control samples of aphid and bacteria.

However, in the second RNA-Seq, RNA was prepared as above (section 5.2) but the treatment regime was modified to a dose of 10^7 CFU mL^{-1} for 38 h. Furthermore mixed RNA from infected aphids was treated to remove aphid 18S & 28S rRNA and polyA tail mRNA for bacteria total RNA enrichment. The final ribodepletion treatment step was carried out on enriched treated and control bacteria RNA to purify mRNA levels. These improved bacterial mRNA preparations were sent for cDNA library preparation and sequencing. For aphid transcriptome profiling high quality total RNA aphid samples (RIN -9-10) were directly sent for library preparation using the TruSeq RNA Library Preparation (Illumina) to capture poly-A mRNA transcripts by Oligo-dT beads, which were subsequently sequenced.

The cDNA libraries were prepared by Illumina TruSeq RNA libraries further subjected to paired-end sequencing. Two lanes of the Illumina HiSeq2000 with a 100bp paired-end read metric was used for RNA-Seq and carried out by The Genome Analysis Centre, Norwich. A separate lane for aphids (8 total RNA) and bacteria (8 ribo depleted) contains four replicates of control and treated samples. This strategy aimed to increase sequencing coverage of the bacterial transcripts in treated aphids.

Both sequencing services provided the raw reads after removing barcodes and demultiplexing. Moreover, in the second RNA-Seq all FASTQ files were generated through use of BCL2FASTQ version 1.8.4 software from the Illumina sequencers. These Illumina FASTQ sequence files were demultiplexed by the sequencing service and also trimmed to a minimum read length of 101bp with a phred quality cut-off of 30 which had a base call accuracy of 99.9 %.

The quality control fastqc tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check all raw paired illumina reads and reported basic statistics of each read. This report comprised data of per sequence GC content, per base quality score, adapter/Kmer content, overrepresented bases and others sequence quality scores. All illumina reads scored under "GOOD ILLUMINA" data with no adapter content and high quality scores.

5.6 Transcriptomic analysis method

As described in the methodology, the sequencing provider and some of mRNA processing steps used in the two RNA-Seq experiments were different, however, in both cases the Tuxedo pipeline was used to count transcript reads. The Tuxedo protocol begins with raw sequencing reads, which detect differential transcript abundance between control and treatment samples (Trapnell *et al.,* 2011) (Figure 5.2).

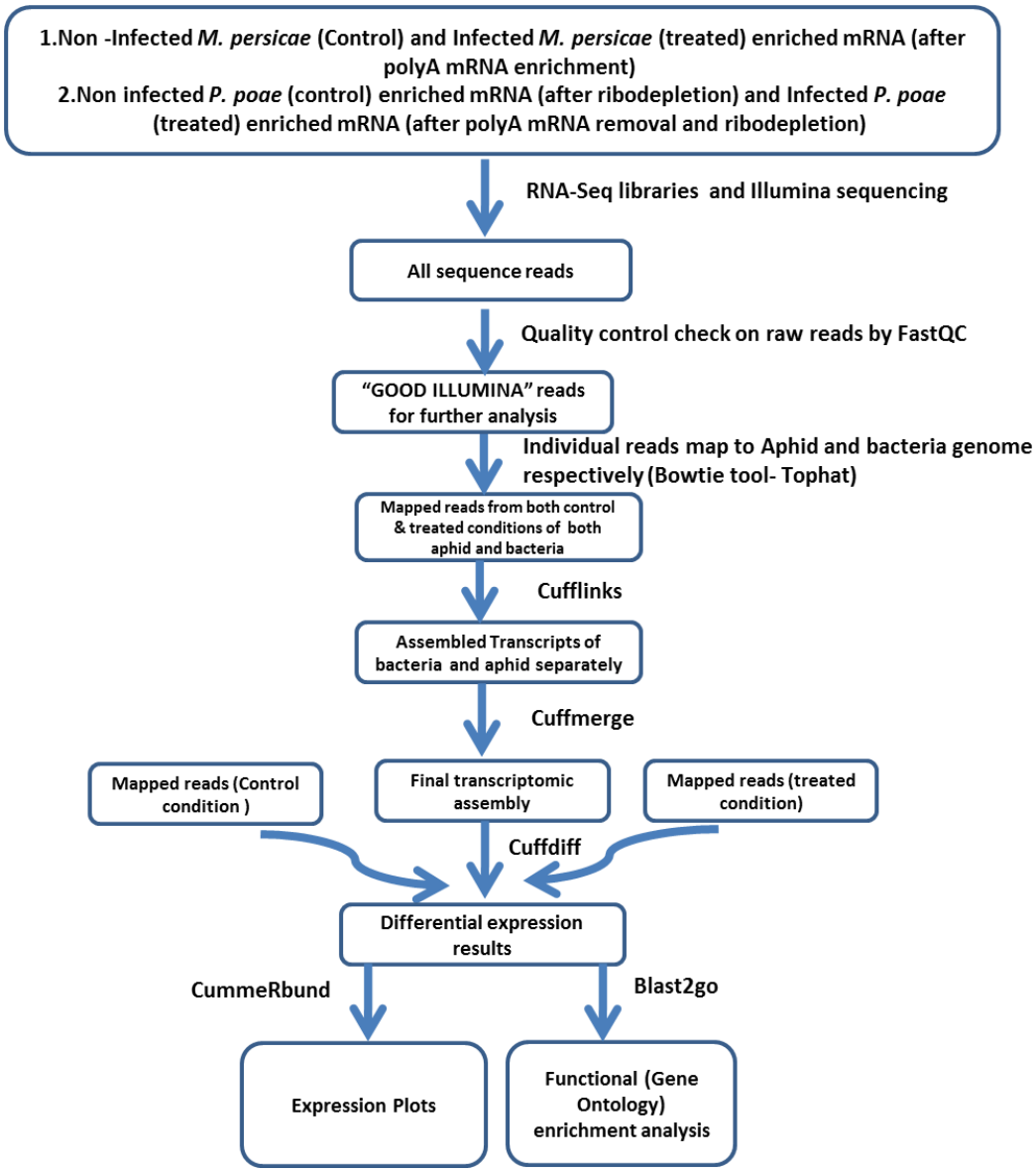


Figure 5.2: The RNA-Seq analysis pipeline. Bioinformatics pipeline for sequential mapping and analysis of simultaneous RNA-Seq data.

The steps below were followed to analyse transcriptomic data:

5.6.1 Quality control on reads

Quality control on raw sequence reads was performed using fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). This step provided statistics on quality summary of reads, eliminating the low quality reads and any genetic contaminants. The illumina reads from the first RNA-Seq experiment required the additional step of trimming reads up to 80 bp which was done using the Galaxy software NGS tool; Trimmomatic-MILNEAN operation. All reads were scored as high quality and preceded to genome mapping.

5.6.2 Align sequence reads to a reference genome

The Galaxy software interface was used for further analysis. The paired-end sequencing forward and reverse reads of each replicate were mapped to the reference *Myzus persicae* Clone_G006 v1.0 {available at ArthropodaCyc metabolic database collection (http://arthropodacyc.cycadsys.org/)} and *Pseudomonas poae* genome {NCBI accession no: NIFJ00000000). To align the sequence reads to the respective genome, the TopHat tool (TopHat v2.1.0, http://tophat.cbcb.umd.edu/) with default parameters was used. All the reads were aligned to their respective genome by using the ultra-high-throughput short read aligner Bowtie, and then analysing the mapping results to identify splice junctions between exons. The alignment output (.bam format file) was viewed using the Integrated Genome Viewer (IGV) to visualize read coverage throughout the whole genome.

5.6.3 Assembly of transcripts and merge

All mapped transcripts were further processed by the cufflinks tool, which assembled all transcripts guided by the reference annotation file. “Accepted hits” file was used with default parameters via the Cufflinks tool (Cufflinks v2.2.1, http://cufflinks.cbcb.umd.edu) for assembling the reads mapped to exons and splice junctions into complete transcripts guided through the reference annotation file. Thus, Cufflinks reports a detailed transcriptome assembly of the data. Moreover, the abundance of assembled transcripts was estimated and reported as **f**ragments **p**er **k**ilobase of exon per **m**illion fragments mapped (FPKM) and confidence intervals were estimated for each FPKM. This metric allows the comparison of each gene's transcript abundance across treatments by normalizing abundance in each treatment for the library's sequencing depth. The supplied reference annotation was used by Cufflinks to guide the reference annotation-based transcript assembly.

It is essential to pool the data and assemble it into a comprehensive set of transcripts before proceeding to differential analysis. The improved approach is to assemble the samples individually and then merge the resulting assemblies together. In our experiment, all transcript replicates of both the conditions were merged using the meta-assembler “Cuffmerge”. We have used Reference Annotation Based Transcript (RABT) assembly to merge reference transcripts with sample transcripts, which produces a single annotation file for use in downstream differential analysis (Trapnell *et al.,* 2012) comprising all assembled transcript and gene ids with loci position on the genome.

5.6.4 Differential expression with Cuffdiff

Cuffdiff (Cuffdiff v2.2.1 (http://cufflinks.cbcb.umd.edu/)) used the TopHat2-aligned reads (the accepted hits file) together with the unified transcript model (the 'merged transcripts' file generated by Cuffmerge) to find transcripts exhibiting differential expression between different conditions (control & treated). Cuffdiff calculates expression in two or more samples and tests the statistical significance of each observed change in expression between them. This statistical model used evaluates read count for each gene across the replicates and uses these variance estimates to calculate the significance of observed changes in expression (Trapnell *et al.,* 2013).

In both RNA-Seq experiments, I employed the same approach to calculate gene expression between control and treated conditions of both bacteria and aphid individually. In Cuffdiff the bias correction and multi-read correct option with reference genome were selected to avoid any artefacts.

Numerous output files were generated by Cuffdiff as a result of differential gene expression between two conditions. Gene and transcript expression level changes are reported in simple tabular output files that can be viewed with any spreadsheet application (such as Microsoft Excel). These files contain statistics such as fold change (in log2 scale), P values (both raw and corrected for multiple testing) and gene and transcript related attributes such as common name and location in the genome.

5.6.5 Data visualization

Cuffdiff produced a number of output files that contain test results for changes in expression at the level of transcripts, primary transcripts, and genes by measuring number of FPKM values. These files are indexed and visualized with CummeRbund (http://compbio.mit.edu/cummeRbund/) to facilitate exploration of genes identified by Cuffdiff as differentially expressed, spliced, or transcriptionally regulated. CummeRbund is a powerful plotting tool which transforms output files into R objects with a wide variety of other packages available from Bioconductor (http://www.bioconductor.org/) within the R environment. This R package solution provides functions for creating commonly used expression plots such as volcano, scatter and box plots to visualize multi-layered datasets.

5.6.6 Functional enrichment

Functional annotation is an effective approach to mining genomic data and uses statistical methods to find categorization in functional classes (e.g., metabolic pathways, cellular processes, etc.). An updated annotation of both bacteria and aphid transcriptome was performed using the BLAST2GO (Blast2GO 3.3) platform. This involves searching genes against the GeneBank non-redundant database using BLASTx algorithms and implementing Gene Ontology (GO) annotation using the Swiss-Prot database and InterProScan (Conesa & Gotz, 2008).

Further, functional enrichment analysis was done by Fisher's exact test to identify enriched biological functions in differentially expressed genes.

5.7 Results

5.7.1 Transcriptomic profiling of aphid and bacteria at infected dose 10^2 CFU mL^{-1} for 48 h

In this study, *Pseudomonas poae* can kill aphids in 48 h of infection. RNA-Seq was used to examine altered gene expression of both host and bacteria during infection. In this initial RNA-Seq experiment, I examined the change in gene expression of aphid and bacteria at 50 % of the lethal dose of bacteria i.e. LC_{50} (10^2 CFU mL^{-1} for 48 h, Figure 3.3A) fed to 4106A aphid clone in diet. The ribodepleted RNA was sent to the Centre for Genomic Research - University of Liverpool for sequencing and all raw reads trimmed to 80 bp on the basis of fastqc quality scores. Further, the *Myzus persicae* clone G006b and *Pseudomonas poae* genomes were used to map control & treated aphid and bacteria reads respectively. The mapping coverage indicated 73 % average mapping rate (more than 30 million reads) in both control and treated *Myzus* reads and around 86 % bacteria control (more than 30 million reads) reads mapped to their genome (Table 5.2). Unfortunately, bacteria RNA from infected aphids showed marginal mapped reads of 0.1 % to genome and only 9,425 aligned pairs in the third replicate (Table 5.2). After genome assembly and transcript merging, Cuffdiff calculated the significant changes in gene and transcript differential expressed output files. The aphid altered profiles showed only 22 genes differential expressed at a cut off false discovery rate of 0.1, which primarily include hypothetical proteins and a few stress and cell morphogenesis genes (Appendix Table 1). However, due to low abundance of bacterial transcripts in treated aphids, most bacterial genes showed no gene count or Zero FPKM values which resulted in the calling of only 59 differential expressed genes at a FDR value of 0.05 (Appendix Table 2).

These results suggested that the dose and duration of infection required optimisation in order to improve the number of bacterial reads obtained from the infected host. Therefore, a time course real-time PCR quantification was conducted on treated aphids to calculate the abundance of bacteria transcripts during the infection process and determine the best dose to use for infection and best time to sample.

Table 5.2: Summary of aphid (*Myzus persicae*) and bacteria (*P. poae*) mapped reads at the early stage of infection.			
Condition – Aphids Infected with 10^2 CFU/mL for 48 h			
Sample Description	**Aligned pairs to their genome**	**Reads mapped to genome**	**Percentage of mapped reads**
P. poae Control (Replicate 1)	6,111,664	30 million	84.6 %
P. poae Control (Replicate 2)	5,200,004	32 million	86.3 %
P. poae Control (Replicate 3)	29,070,319	65 million	88.3 %
P. poae Treated (Replicate 1)	21,306	0.05 million	0.1 %
*P. poa*e Treated (Replicate 2)	39,029	0.05 million	0.2 %
P. poae Treated (Replicate 3)	9,425	0.02 million	0.0 %
Myzus persicae Control (Replicate 1)	15,149,877	32 miilion	73 %
Myzus persicae Control (Replicate 2)	18,226,107	42 million	74 %
Myzus persicae Control (Replicate 3)	19,418,134	39 million	75 %
Myzus persicae Treated (Replicate 1)	37,292,249	86 million	75 %
Myzus persicae Treated (Replicate 2)	27,145,659	85 million	69.7 %
Myzus persicae Treated (Replicate 3)	33,187,051	87 million	74 %
The genome mapping showed total aligned pairs with concordant mapping rate of samples in both condition (control & treated) with their all replicates.			

5.7.2 Evaluation of bacteria transcript inside treated aphid samples by real time PCR method

A *P. poae* infection dose of 10^7 CFU mL^{-1} in Mittler diet caused death of aphid clone 4106A in 48 h (Figure 3.2A). Therefore, to quantify bacterial transcripts inside aphids during the course of infection, I employed Quantitative PCR (QPCR) to calculate the expression of bacteria mRNA levels in heterogeneous aphid – bacteria RNA samples. QPCR was conducted on aphid treated cDNA samples after infection of bacteria at 12, 20, 24 and 36 h and the amount of bacterial transcripts of *P. poae* measured using specific housekeeping and toxin primers The results showed that no bacterial transcripts were recovered at the 12 h infection time but after 20 h of bacterial treatment there was a constant increase in bacterial transcripts. Cycle threshold (Ct) values observed in QPCR reflect the bacterial cDNA levels with Ct values falling during the course of infection (Table 5.3).These data helped identify the optimal infection time point for further RNA profiling experiments. After 38-40 h *P. poae* infection, most aphids started melanisation along with reports of 20 -30 % aphid death in treated sachets. Hence, using a higher infection dose after 38 h to increase bacterial numbers will have a corresponding effect with an increase in mortality of aphids. This would compromise the expression profiles obtained from aphids; therefore, I considered 38 h as optimal as up to this infection time point

no aphid mortality was observed. To further increase the sequencing of bacterial transcripts in treated aphids I also employed an mRNA bacterial enrichment method in mixed aphid-bacteria RNA samples.

Table 5.3: Assessment of bacteria transcript inside aphids by time course quantitative PCR.

	***rpoD* gene (Housekeeping)**		***efeOB g*ene (Iron Transport)**		***tcaA* gene (Toxin)**	
Treatment time (hpi - h post infection)	**Cycle Threshold (Ct –values)**	**Standard Error**	**Cycle Threshold (Ct –values)**	**Standard Error**	**Cycle Threshold (Ct –values)**	**Standard Error**
20hpi	31.95	0.34	29.70	0.40	34.94	0.11
24hpi	27.385	0.36	25.13	0.61	32.46	0.24
36hpi	23.335	0.15	20.26	0.52	30.97	0.32

QPCR conducted on bacteria treated aphid cDNA after infection of 20, 24 & 36 h and the level of bacteria transcript measured by housekeeping and other genes. The data presented are the mean and standard error of four biological replicates.

To conclude, study of infection through real time PCR and bacteria growth assay inside aphids, suggested a highest infected dose of 10^7 CFU mL^{-1} in Mittler diet and an optimal time point for sampling of 38 h. This optimum treatment strategy was used for a second RNA sequencing experiment to investigate altered host-pathogen expression upon ingestion of virulent bacteria.

5.7.3 Simultaneous transcriptional profiling of aphids and bacteria at critical dose 10^7 CFU mL^{-1} for 38 h

5.7.3.1 Genome mapping and Transcript assembly

The Tuxedo pipeline was followed for expression analysis as detailed above. All raw reads of aphid and bacteria were mapped to their respective genome using the bowtie aligner in TopHat. The genome mapping results showed the coverage of *Myzus* in both control and treated conditions was ~89 % in all replicates (Table 5.4). All aphid mapped transcripts were assembled by Cufflinks and merged by the assembler "Cuffmerge" to generate 18,400 genes with 32,184 transcripts. Similarly, all bacteria control replicates also showed 89 % mapped reads to genome although lower mapping rate (~1 %) was observed in treated enriched mRNA of bacteria. 4,612 genes and 5,467 transcripts were called after assembly and merging of bacteria transcriptome. Besides these, bacteria-treated reads were mapped to the *Buchnera* genome with 13 % in all replicates. Due to the absence of control *Buchnera* mapped reads, no further transcriptomic analysis was conducted.

The mapping results showed that all aphid replicates of both conditions had more than 35 million reads mapped to the *Myzus persicae* genome, resulting in good RNA Seq coverage as per guideline mentioned in section 5.5. Similarly, bacterial control transcripts also represented excellent RNA Seq coverage with more than 8 million reads. However, mapped reads of bacteria treated samples were still low which required more quality control check for further analysis.

Table 5.4: Summary of aphid (*Myzus persicae*) and bacteria (*P. poae)* mapped reads at infected dose 10^7 CFU $mL^{-1}$for 38 h.

Condition – Aphids Infected with 10^7 CFU mL^{-1} for 38 h			
Sample Description	**Aligned pairs to their genome**	**Reads mapped to their respective genome**	**Percentage of mapped reads**
P. poae Control (Replicate 1)	19,081,679	22.3 million	93.3 %
P. poae Control (Replicate 2)	21,658,693	19 million	86.1 %
P. poae Control (Replicate 3)	22,251,327	23 million	90.8 %
P. poae Control (Replicate 4)	24,868,693	25 million	93.8 %
P. poae Treated (Replicate 1)	247,565	0.6 million	1.1 %
P. poae Treated (Replicate 2)	234,502	0.7 million	1.1 %
P. poae Treated (Replicate 3)	401,474	0.8 million	1.4 %
P. poae Treated (Replicate 4)	315,879	0.85 million	1.2 %
Myzus persicae Control (Replicate 1)	22,698,481	35 million	89.7 %
Myzus persicae Control (Replicate 2)	14,748,106	38 million	89 %
Myzus persicae Control (Replicate 3)	17,089,871	40 million	89.7 %
Myzus persicae Control (Replicate 4)	19,572,092	36 million	90.4 %
Myzus persicae Treated (Replicate 1)	18,763,846	42 million	89.7 %
Myzus persicae Treated (Replicate 2)	19,284,701	38 million	89.4 %
Myzus persicae Treated (Replicate 3)	16,818,149	39 million	89.7 %
Myzus persicae Treated (Replicate 4)	19,007,351	38 million	86.7 %

5.7.3.2 Differential expression analysis and Data visualization

The Cufflinks and Cuffdiff output was examined for fidelity with several quality control methods. First, the variation between the replicates and both conditions was assessed with a Multidimensional Scaling (MDS) plot (Figure 5.3). In the MDS plot, the biological replicates of control (both in aphid & bacteria) clustered closely, indicating that there was little variation among the replicates. While in case of treated conditions (both in aphid & bacteria) three replicates clustered with small variations while a fourth replicate deviated from others. This variation might be the effect of treatment during the course of infection (Figure 5.3).

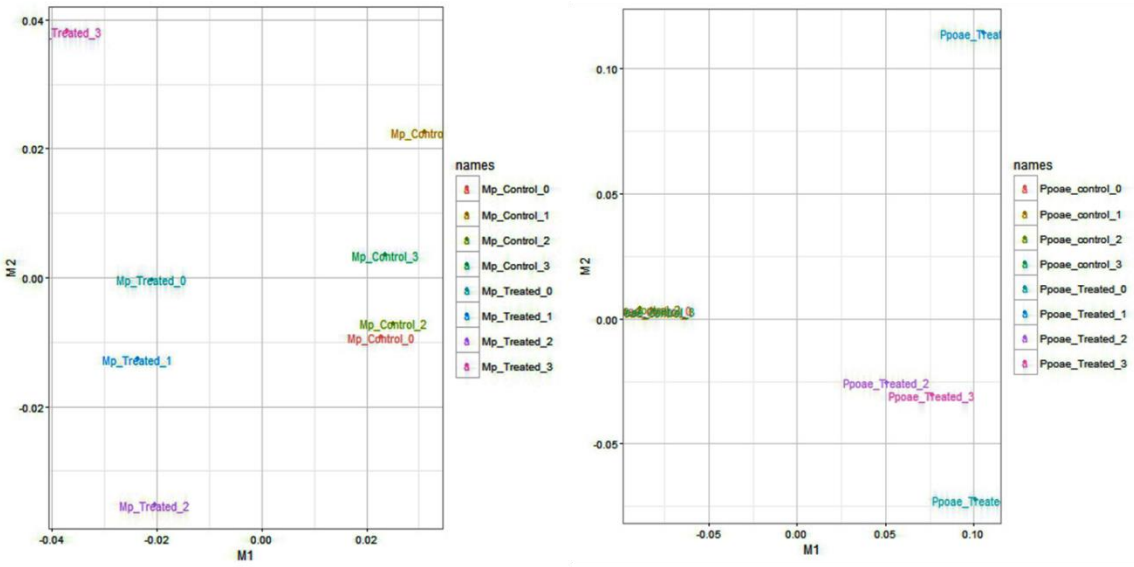


Figure 5.3: Multidimensional scaling plot of samples based on genes found to be differentially expressed (DE) between two conditions.

The dynamic range of the FPKM values was also evaluated by using CummeRbund to create a boxplot & csdensity of log_{10} transformed FPKM values for both conditions. The overall range and quartile distribution were consistent among conditions of both aphid & bacteria, indicating that the data were reproducible and of high quality (Figure 5.4 and 5.5).

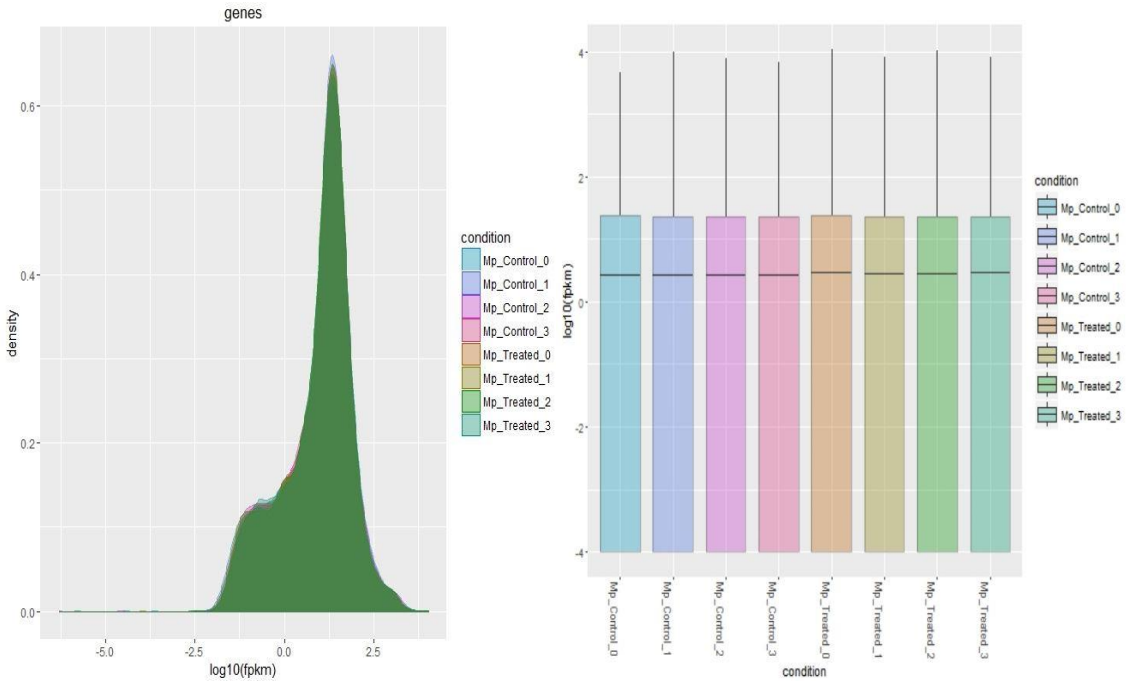


Figure 5.4: (A) Density plot displaying the number of genes at each mean FPKM value from all replicates of control and treated *M. persicae* conditions. The solid bold line highlights the mean FPKM less than Zero, indicating that very high levels of sequence coverage allowed the identification of genes with very low levels of expression.

Figure 5.4: (B) Boxplots display the range of FPKM values of all *M. persicae* genes surrounding the mean fragments per kilobase of exon per million fragments mapped (FPKM) value for each replicates of control and treated *M. persicae* conditions. Dynamic range of FPKM values represented as log_{10} transformed FPKM values for each gene for all replicates of both conditions showed no deviation.

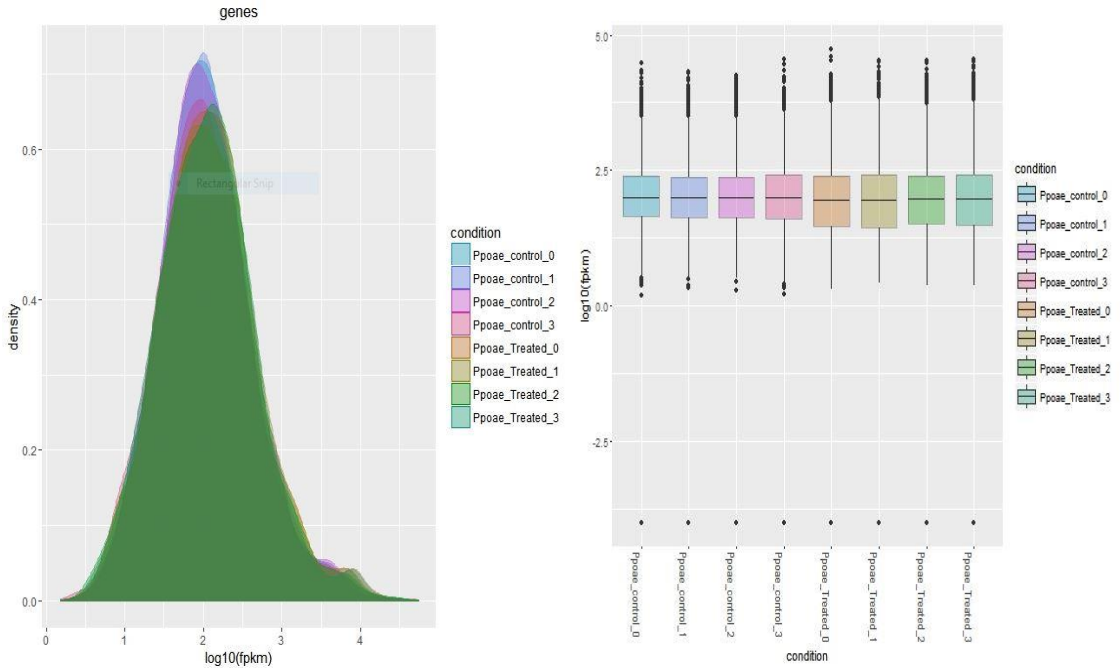


Figure 5.5: (A) Density plot displaying the number of genes at each mean FPKM value from all replicates of control and treated *P. poae* conditions. The solid bold line highlights the mean FPKM minimum cut-off of 5.0 used in the subsequent analysis of differential gene expression.

Figure 5.5: (B) Boxplots display the range of FPKM values of all *P. poae* genes surrounding the mean FPKM value for each replicates of control and treated *P. poae* conditions. Importantly the data confirmed that there was no inflation of values between control and treated reads as a consequence of differences in sequence depth.

The aphid Cufflinks datasets indicated median FPKM values among conditions were similar and slightly less than 1, indicating that very high levels of sequence coverage allowed the identification of genes with very low levels of expression. Importantly, the low number of reads in the treated bacteria sample did not bias the FPKM counts in regard to the control; hence a minimum cut off FPKM value of 5 was used for subsequent analysis. Around 95 % of genes in all replicates of treated bacteria consistently showed FPKM of more than 10 which did not interfere with the differential gene analysis due to low sequence depth.

A volcano plot describes the relationship between statistical significance for tests of DE and relative transcript abundance for both conditions. The *Myzus*_Treated/*Myzus*_Control plot shows that the equal percentage (50 %) of DE genes were regulated both sides between *Myzus* control & treated, similarly the total number of DE genes were 2094 and around 47 % DE genes were upregulated with the rest downregulated between *P. poae*_Treated/*P. poae*_Control (Figure 5.6).

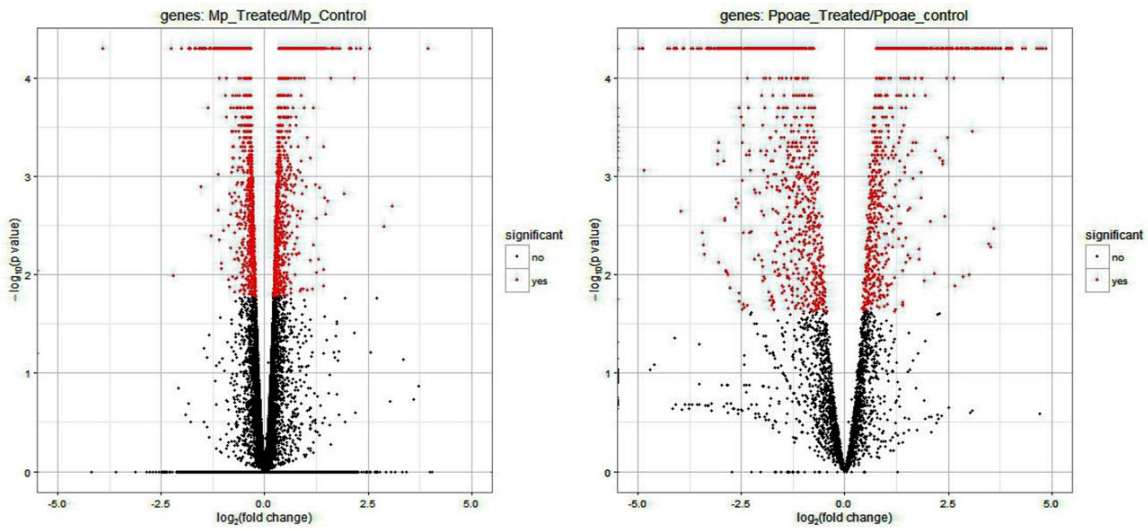


Figure 5.6: Volcano plots of *M. persicae* and *P. poae* transcriptomic data. The plots showing the relationship between statistical significance of each test for Differential expressed (DE) and relative transcript abundance for both *M. persicae* (aphid) and *P. poae* bacteria conditions. Significant DE genes are coloured red.

Cuffdiff provides analyses on FPKMs for each transcript which were summed across all transcripts associated with each gene to produce the abundance metric for testing DE at the gene level. P values were estimated for each gene and corrected for multiple testing (q value) by Benjamin-Hochberg correction. For all significant tests ($q \leq 0.05$) the sign of the $\log_2$ (fold change) was used to partition the DE genes into up and down regulated groups. The differential expressed gene profiles of aphid and bacteria were 3220 and 2094 genes respectively using a cut off P value of 0.05.

To understand large sets of DE genes with their related biological function, further visualization of data were categorized into “aphid” and “bacteria” transcriptomic profiling. This allows categorization of genes in functional classes, which can be very useful to understand the physiological meaning of large numbers of DE genes and to assess functional interaction between aphid and bacteria.

5.7.4 Aphid transcriptomic profiling

To examine only biological relevant interesting gene changes we selected more than 2-fold changes (in both up and down regulation) on differential expressed (DE) genes for subsequent analysis. This reduced the number of DE genes in aphids to 193 of which 112 and 81 were upregulated and downregulated respectively (Figure 5.7). The upregulated gene profiles of annotated gene (100) genes varied in their expression from 2-4-fold. Of the 81 genes that were downregulated 5 genes were reduced by >4 fold and the remainder decreased by fold changes of ~2-4.

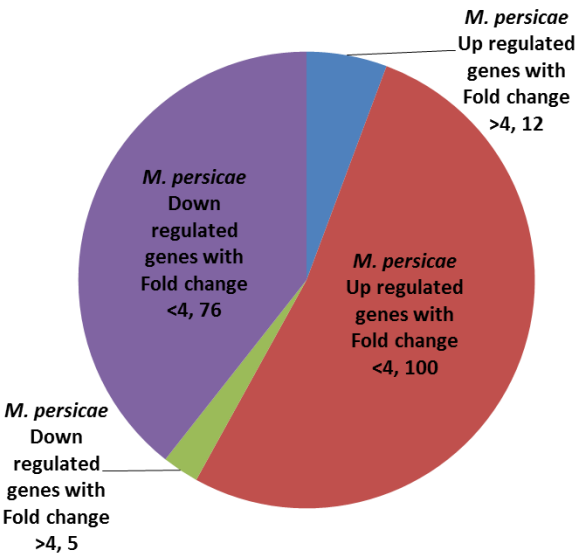


Figure 5.7: Differential expressed genes in *M. persicae* transcriptomic data. Pie chart displays number of differential expressed genes more and less than fourfold change in both direction of regulation in aphid altered profile data at P value of 0.05.

5.7.4.1 Differential expression of immune, apoptosis and stress genes

During infection of *M. persicae* by *P. poae*, upregulation of two cuticular proteins, chemoreceptor protein (take out) and three innate immune protein motifs such as sterile alpha and TIR motif, leucine rich repeat and Pv-fam-d protein by 2-3 fold was observed in treated aphids (Appendix Table 3 A). Genes which were involved in the cellular uptake of xenobiotics (receptor - nose resistant to fluoxetine receptor of saliva) and (transporter–trehalose) were increased by 2-fold (Bansal *et al.,* 2014). The upregulation of redox gene dehydrogenase reductase SDR family by 2-fold was also observed (Appendix Table 3A).

However, there was also evidence of down-regulation of a number of defence and immunity proteins inside aphids. During infection by *P. poae,* expression of aphid lysosome genes such as *carboxylesterase E4-like*, *sulfotransferase lysosomal Pro-X carboxypeptidase* were downregulated by 2-3-fold (Bansal *et al.,* 2014; Ye *et al.*, 2014). The major antioxidant enzyme of saliva named "*gamma-glutamyltranspeptidase 1-like*" was reduced by 15-fold along with xenobiotic metabolism genes like *UDP-glucuronosyltransferase, esterases and cytochrome b561-like* also downregulated, by 3-4 fold. Reduced expression of stress genes such as *heat shock 63, dehydrogenase reductase SDR family member 11-like* and peroxidases were also observed (Ye *et al.,* 2014) (Appendix Table 3 A).

The genome annotation identified apoptosis genes named '*centromere associated E'* and *'comm3 isoform D'* were overexpressed by 3-fold and *'negative regulator of neuron apoptotic process legumain isoform X1'* was downregulated by 2.8 fold (Appendix Table 3 A).

5.7.4.2 Differential expression of proteases and other digestive enzymes

RNA-Seq analysis revealed 5 protease-related genes showing higher expression and 4 proteases showed reduced expression in treated aphid profiles compared to controls. All putative protease genes with higher transcript levels were most similar to cysteine proteases, and were named *cathepsin B-N* & *TPA_inf: cathepsin B* (Rispe *et al.,* 2008). The serine protease (venom protease) and matrix metalloproteases are involved in defence mechanisms (Kutsukake *et al.,* 2004). The transcript levels for these genes exhibited an increase in treated aphids ranging from 2-3 fold (Appendix Table 3 B).

The putative protease genes with lower transcript levels in treated aphids included four genes similar to *cysteine proteases* and *aminopeptidase N-like* with reductions in expression of 2-fold and 3.5-fold respectively(Bansal *et al.,* 2014) (Appendix Table 3 B).

Functional annotation revealed transcripts encoding two forms of serine protease inhibitor; *regucalcin-like isoform* and *angiotensin-converting enzyme-like isoform X3* upregulated 2.2 and 4 fold respectively (Bansal *et al.,* 2014) (Appendix Table 3 B).

Other digestion enzyme such as leucyl-cystinyl aminopeptidase, lipases and mucin-like were also upregulated (Appendix Table 3 B).

5.7.4.3 Differential expression of cell locomotion and cytoskeleton genes

The myofibrillar gene "*titin*" involved in flight and muscle contraction increased in expression by 2.28- fold along with expression of muscle contractile regulator "*PDZ and LIM domain Zasp isoform X12*" (2 fold) (Sinha *et al.*, 2016) (Appendix Table 3 C). The elevated levels of major chaperone protein "*Tubulin-specific chaperone cofactor E*" by 2.44-fold, which is required for the normal development and function of neuromuscular synapses, was observed (Appendix Table 3 C). The *nesprin* gene associated with nerve cell cytoskeleton was also upregulated by 2.3-fold (Morel *et al.,* 2014).The decreased level of gene *kintoun* which is required for cytoplasmic pre-assembly of axonemal dyneins by 2-fold was observed (Von Morgen *et al.,* 2015) (Appendix Table 3 C).

5.7.4.4 Differential expression of various cell communication pathways

The genes associated with cellular proliferation and differentiation process including *Noggin*, regulator of rho and phosphatidylinositol signalling were upregulated in treated aphids (Bond *et al.,* 2012). The various transcription factors potentially related to signalling of these processes, such as *Zinc finger MYM-type 1-like*, *Zinc finger 271-like*, *yippee-like 1, suppressor SRP40-like* and *homeobox engrailed-2-B,* were also overexpressed by 2-2.5-fold (Peel *et al.,*

2006; Hosono *et al.*, 2010; Salvemini *et al.,* 2013)(Appendix Table 3 C).

The reduced expression of cellular signalling receptor such as somatostatin and olfactory receptor were reported. Two signal molecules defence Hdd11 and craniofacial development 2-like partial, which are required for nervous development and cell proliferation, were downregulated by 2- and 3- fold. The gene associated with adrenergic receptor signalling pathway '*arrestin domain-containing protein 3*' was also decreased by 2.5-fold (Puca & Brou, 2014) (Appendix Table 3 C).

5.7.4.5 Changes in transporter activity

A 2-fold increased expression of ion channels such as cationic amino acid, sodium & solute carrier anion, sodium potassium calcium exchanger 4-like and sodium channel which are involved in transporter and nerve transmission activity were observed (Appendix Table 3 D).

In contrast a 2.5-fold drop in expression of major facilitator superfamily and solute carrier family gut transporters was seen. These transporters facilitate the reduction of amino acid, sugars and lipid absorption in midgut. A 4-fold reduced expression of *trehalose transporter*, which is involved in removal of foreign radicals from haemolymph, was also observed (Appendix Table 3 D).

Other peptide and proton-coupled amino acid transporters were also downregulated by 2.2-fold as were Iron transfer protein ZIP transporter of mid gut and nerve transmission acetylcholine receptor by 2.5 fold (Appendix Table 3 D).

5.7.4.6 Alteration in protein ubiquitination, endocytosis and protein trafficking process

A few genes associated with protein trafficking and proteolytic processes were differentially expressed between control and treated aphids. The upregulation of ubiquitination gene *cysteine and histidine-rich 1 homolog* & probable *GPI-anchored adhesin PGA55* by 2.18-folds and downregulation of two deubiquitinating gene *ring canal kelch homolog* by 2.26-fold were observed (Rosenbaum *et al.,* 2013) (Appendix Table 3 C).

The protein trafficking regulator Zinc finger 521 was increased by 2-fold and arrestin domain-containing 3-like, which is involved in trafficking and ubiquitination, were downregulated by 2.5-fold in infected *M. persicae* compared to controls.(Appendix Table 3 C).

5.7.4.7 Effect on metabolism

A few genes involved in catabolism of amino acids (tyrosine, proline) and sugars (glucose) were overexpressed in treated aphids between 2-3 fold (Appendix Table 3 E).

The downregulation of the below genes involved in metabolism were observed in altered aphid profiles:

- "*Carotenoid desaturase*" and "*lycopene" cyclase* which are involved in carotenoid biosynthesis pathway were decreased by 39-fold and 2.2-fold respectively.
- *Omega-amidase NIT2* gene expression, which is involved in nitrogen metabolism to remove toxic intermediates.
- Enzymes involved in amino acid metabolism such as *Gamma-glutamyl hydrolase A*-like- (glutamine), *Homocysteine S-methyltransferase 1-like*- (Cysteine and methionine) and *Enolase-phosphatase E1* (methionine biosynthesis via salvage pathway).

Finally several hypothetical and other uncharacterised genes DE in both directions ranging from 2-15 folds were also observed (Appendix Table 3F).

5.7.5 Bacteria transcriptomic profile

As a result of Cuffdiff analysis, *P. poae* showed 46 % of total genes differential expressed between treatment and control which were further subjected to fold change (>2) filter to provide 1325 bacteria DE genes (representing 24 % of the total gene count of this bacteria). Of these 542 were upregulated and 783 down regulated (Figure 5.8). In the upregulated gene list, 121 genes showed a fold change of more than 5 and 421 genes <5-fold whereas, 155 genes were downregulated by >5 fold and 628 genes less than 5-fold.

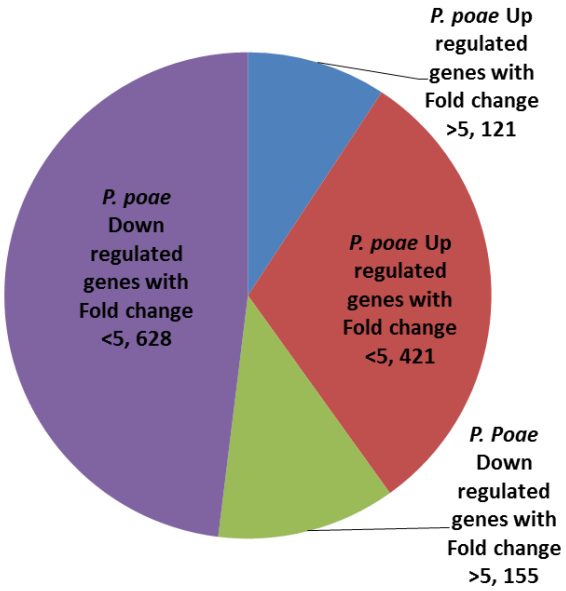


Figure 5.8: Differential expressed genes in *P. poae* transcriptomic data. A pie chart displaying the number of genes differentially expressed more and less than fivefold in either direction between control and treatments at P value of 0.05.

5.7.5.1 Upregulation of iron acquisition system and transporter across membrane

Genes for the biosynthesis and uptake of the siderophores pyoverdine were more highly expressed in treated bacteria. Within the up-regulated pyoverdine biosynthesis gene clusters, the most highly expressed gene was *pvdS,* which encodes the Extra-Cytoplasmic Function (ECF) sigma factor *pvdS*, a transcriptional regulator of pyoverdine biosynthesis gene. In this study, all genes directly downstream of a putative PvdS-controlled promoter region were transcriptionally up-regulated by more than 5-fold (Appendix Table 5A).

In addition to Siderophore, haem acquisition systems were also upregulated by more than 10fold and '*haem oxygenase'*, involved in haem degradation to release bound iron increased in expression by 58-fold along with associated sigma factor and transporter proteins. Several encode ECF sigma factors, many of which are likely to control iron homeostasis and heterologous ferric-siderophore complexes, TonB-dependent receptors were also

overexpressed by 5-12-fold. Iron transport across the membrane by divalent metal uptake transport (Zn, Cu, Ni) also increased by 2-4-fold. Additionally, transport of ferrous iron into the cytoplasm by the EfeUOB system was also upregulated by more than 15-fold (Appendix Table 5A).

5.7.5.2 Differential activity of other transporters

Increased expression of 39 different kinds of ABC transporters by more than 10-fold was observed which includes those transporting dicitrate, phosphate, molybdenum, dipeptides, amino acids, sugars, urea and polyols (Appendix Table 5C). However, the downregulation of several antibiotic efflux systems such as the five multidrug resistance efflux pumps (Cme A, B) RND protein, three permeases of the major facilitator superfamily and 3 Permease of the Drug/Metabolite Transporter (DMT) superfamily by more than 10-, 30-, and 3-fold respectively was also observed. Other transporters including those transporting nitrate/nitrite, alkanesulfonates, taurine, xanthine and amino acid were decreased by 2.5-4-fold (Appendix Table 5C).

5.7.5.3 Transcription of virulence factors, toxins and oxidative stress

The expression of virulence factors like type IV secretory proteins and alkaline metalloproteinase (aprX) increased by 3-5-fold in treated bacteria, whereas downregulation of genes associated with pathogenicity was seen in treated bacteria such as *tcaC* subunit of Tc toxin and Rhs family proteins by 2.28- and 4.3-fold respectively (Appendix Table 5D) (Vodovar *et al.,* 2005; Yang & Waterfield, 2013). The upregulation of defence mechanisms against oxidative stress was suggested in treated bacteria as the expression of the antioxidant genes Glutathione S-transferase, manganese superoxide dismutase and thiol peroxidase2c Tpx-type was upregulated (Appendix Table 5D).

5.7.5.4 Changes associated with metabolism

The genes associated with lipopolysaccharide, lipoprotein and fatty acid biosynthesis and Inositol catabolism were overexpressed by 2-3-fold in treated bacteria (Appendix Table 5B). In carbohydrate metabolism, lower expression of citric acid genes and activation of glyoxlate cycle genes (for utilization for simple carbohydrates) were observed (Appendix Table 5B). Several transcription regulators related with stress (Sensory PhoP & sigma factor RpoS), iron (Sigma factor2C ECF subfamily), phosphate (PhoB) (SphR) and biosynthesis pathways (Transcriptional regulator2C GntR family & glmS gene2C DeoR family) were induced (Appendix Table 5G). Various catabolic reactions like degradation of fatty acids, amino acids (lysine, arginine, glutamine & aromatic amino acid) were downregulated by 2-3-fold (Appendix Table

5G). The genes (TetR, LysR, AraC, and GntR) which are involved in the transcriptional control of multidrug efflux pumps, pathways for the biosynthesis of antibiotics, response to osmotic stress and toxic chemicals, control of catabolic pathways, differentiation processes, and pathogenicity were also downregulated (Appendix Table 5G).

5.7.5.5 Transcription of flagella and adhesion genes

Downregulation of 26 genes associated with adhesion (Pilli and fimbriae) and their assembly proteins was observed by 3-10-fold. The differential expression of seven methyl chemotactic genes was observed out of which five were upregulated and rest downregulated by 2-2.5-fold (Appendix Table 5E). The elevated level of flagellar biosynthesis genes (f*lgB, flgC, flgE and flaB*) with upregulation of two Pilli assembly proteins by 2-fold was also observed (Appendix Table 5E).

5.7.5.6 Iron starvation stress responses

The downregulation of various genes associated with sulphur assimilation (taurine, alkanosulphonates), respiratory chain gene clusters (*cytochrome oxidase*), and oxidative stress (*catalase, alkyl hydrogen peroxide reductase, super dismutase-Fe*) allowed the bacterium to conserve iron demands on the cell (Appendix Table 5B) (Appendix Table 5D) (Appendix Table 5G). DNA repair and recombination genes expression are regulated by OxyR regulon (iron regulated) and showed lower expression by fold change ranging from 5-20. (Appendix Table 5G).

5.7.6 Functional annotation of differential expressed genes by Blast2go

Blast2go (v3.3) was used to gain biological insight from DE gene lists and to identify enriched Gene Ontology (GO) terms, and find functionally related gene groups. Significantly up and downregulated gene lists from each comparison were submitted to the functional annotation tool and analysed with the functional pathway options.

In *Myzus* DE genes, the majority of transcripts assigned to the ‘biological process’ domain were involved in cellular, regulatory, developmental, and biosynthetic activities (Figure 5.9). The transcripts under ‘molecular function’ domain were predicted to have catalytic, binding and transporter functions. Four GO terms associated 'cellular components' such as Arp2/3 protein complex, nuclear part, Voltage-gated sodium complex and endomembrane system were reported.

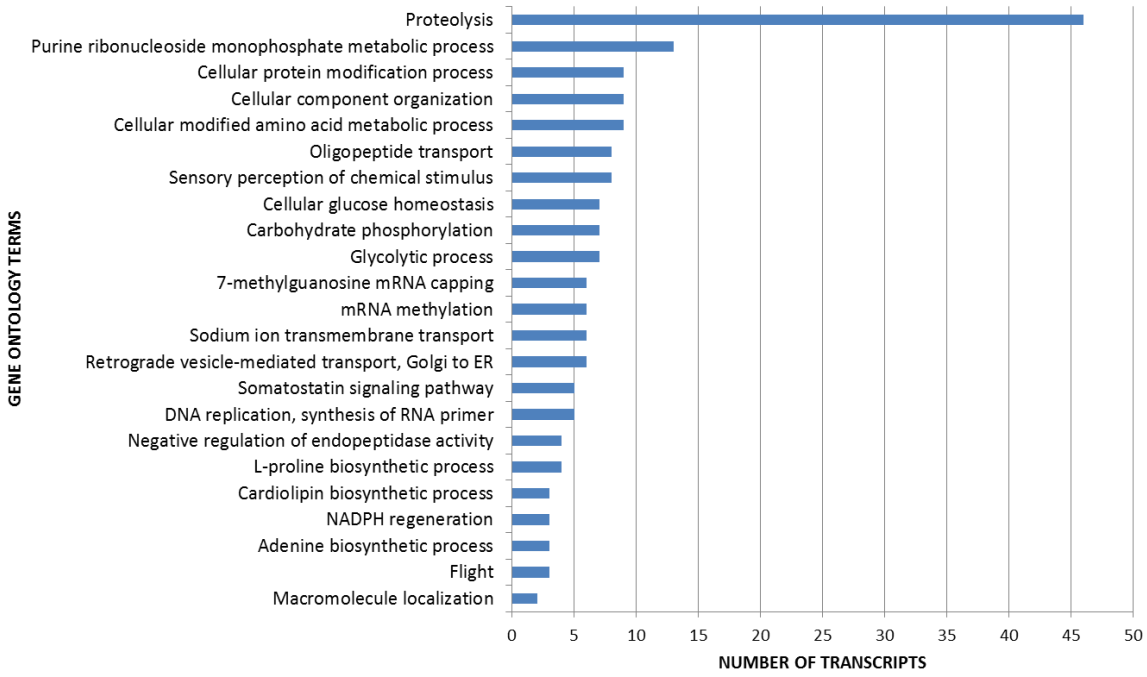


Figure 5.9: Biological process graph for GO terms annotated with BLAST2GO (*M. persicae*). The bar graph is depicting "biological process" category analysis of the *M. persicae* differential expressed genes.

In the bacterial transcriptome, the biological process of upregulated transcripts was assigned to transport process, metabolism of polyol & nucleic acids along with regulators of metabolic process. Similarly bioprocess of downregulated transcripts were assigned to pathways for metabolism of nitrogenous compounds (e.g. purine, pyrimidine, amino acids) and sugar along with regulators of transcription & translation process (Figure 5.10). The molecular function associated to phosphopantetheine, nucleic acid binding, transfer activity along with external encapsulating structure, intracellular ribonucleoprotein complex, organelle part terms associated with Cellular Component were assigned to certain bacteria transcripts.

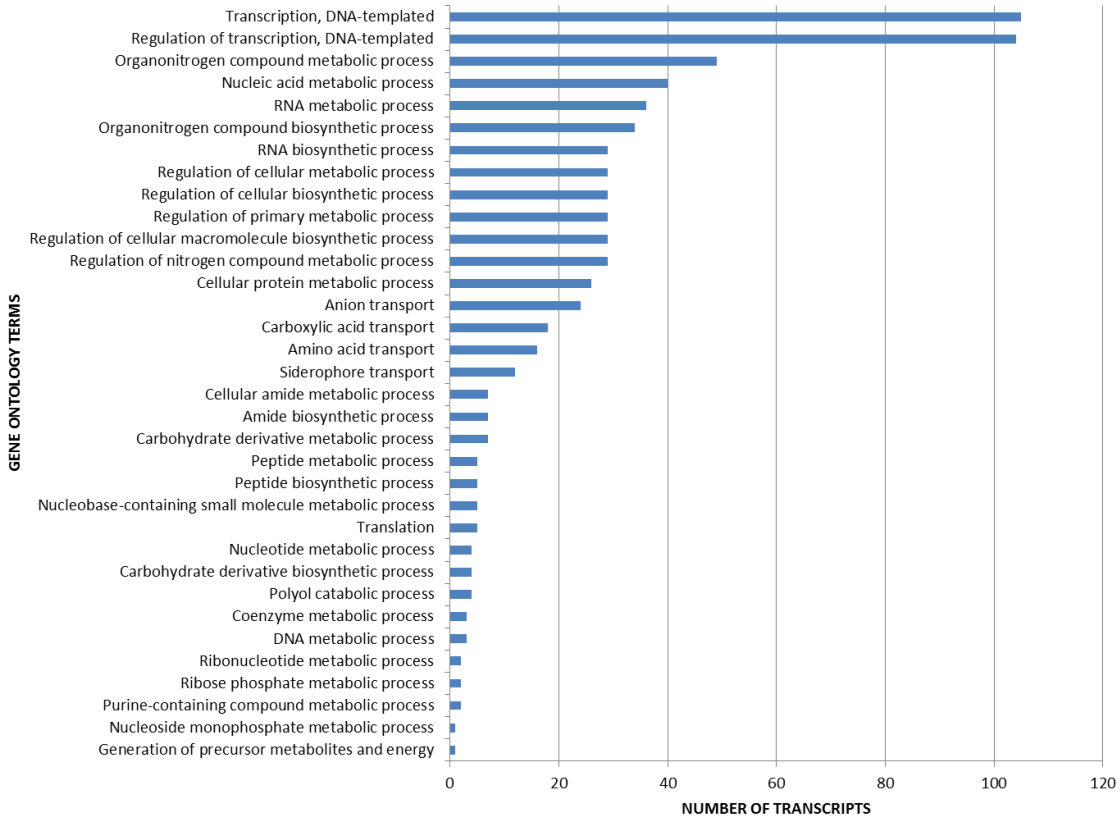


Figure 5.10 Biological process graph for GO terms annotated with BLAST2GO (*P. poae*). The bar graph is depicting "biological process" category analysis of the *P. poae* differential expressed genes.

To further understand the genome-scale data, I explored which biological functions are enriched in lists of DE genes. Functional enrichment analysis was conducted using the Fisher Exact test tool of Blast2go (Blast2GO 3.3).

Functional enrichment analysis of *Myzus* DE genes (at FDR 0.025) showed 50 GO terms enriched out of which 44 GO terms were over-enriched and 6 GO terms were under-enriched (Figure 5.11). The enrichment of bioprocess such as somatostatin signalling, DNA replication, mRNA processing, glycolysis, adenine biosynthetic process and L-proline biosynthetic process are with 1-2.5 % percentage of test genes. Sensory perception, proteolysis and glucuronosyltransferase activity were majorly enriched with more than 2.5 % of test DE genes. Two molecular functions RNA binding and cellular protein modification process along with cellular components of endomembrane system were underrepresented in enrichment analysis.

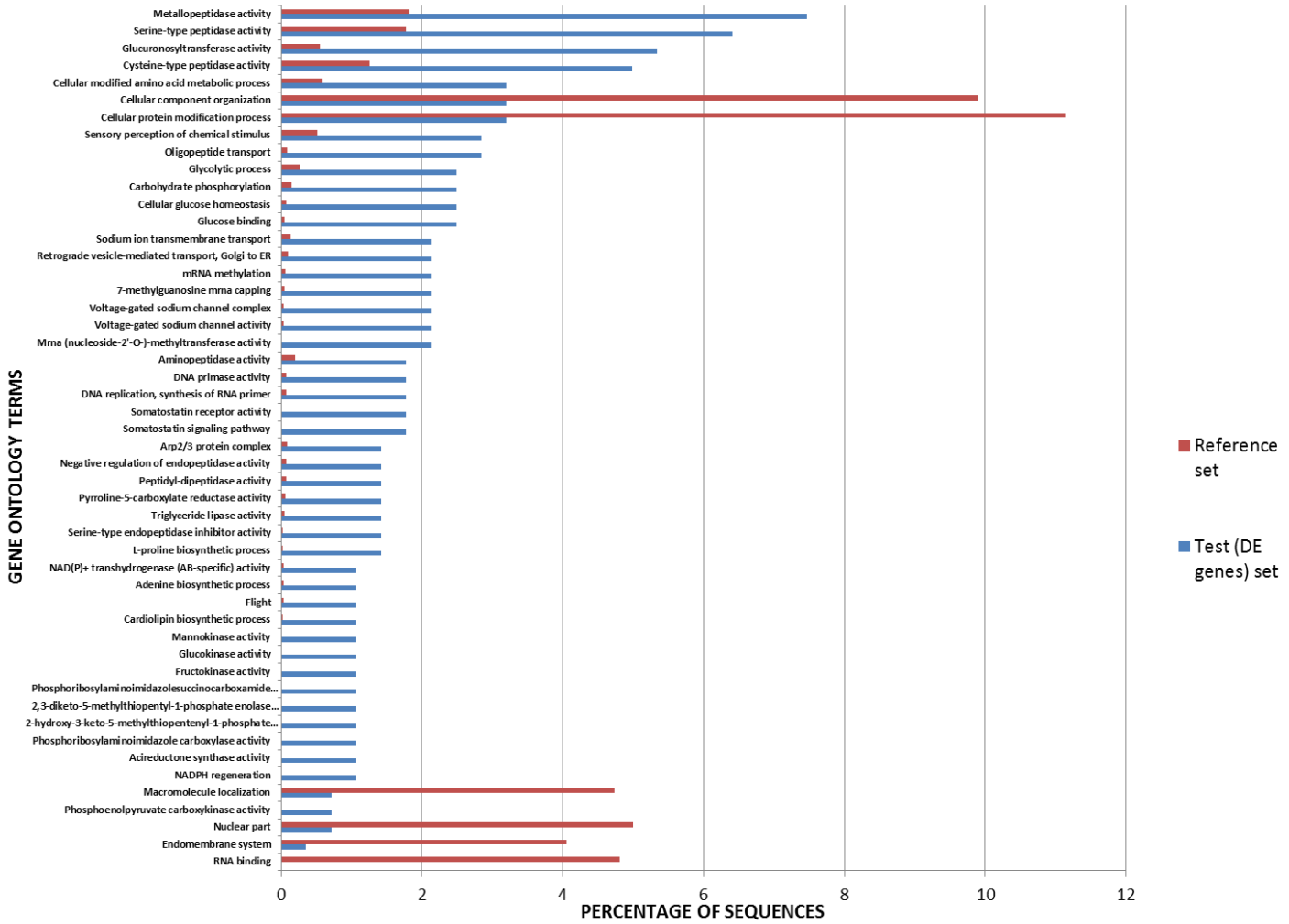


Figure 5.11: GO term enrichment analysis on *M. persicae* transcriptomic data. The figure represents all the significant GO-term categories found significantly enriched compared to the reference set (all genes present on the RNA-Seq data) after a Fisher's exact test and Benjamini and Hochberg multiple testing correction (FDR,0.05). The test set percentage indicates the percentage of differential regulated genes belonging to a GO term category compared to all differential expressed genes used in the GO-term analysis while the reference set percentage indicates the percentage of a particular GO-term category compared to all genes with GO-terms on the RNA-Seq data.

In the bacterial analysis, I used a separate Fisher exact enrichment test on up and down regulated gene sets to identify major enriched process in large sets. The overexpressed DE genes showed 44 GO Terms (FDR 0.025) which were divided into 24 over and 20 under enriched GO terms (Figure 5.12). All cell components terms of upregulated genes were over enriched by 3 – 6 % of test genes. The over enrichment of general transport and ion transport by 25 % and 10 % test genes respectively followed by various transporters (amide, anion, carboxylic and amino acid) with 3.5 – 6 % test genes. The iron uptake process (Siderophore, iron chelate transport & iron coordination activities) and binding (phosphopantetheine, vitamin & amide) were over enriched with 2-4.5 % test genes. Several additional metabolic process such as RNA, nucleobase-containing compound, cellular aromatic compound, heterocycle and organic cyclic compound metabolic process were under-represented.

In the downregulated DE gene lists, a total of 70 GO terms were enriched (Figure 5.13). 25 mainly comprised transcription factor activity, DNA & RNA binding and different biological regulation processes showed over enriched with maximum 18-19 % value of test DE genes. The remaining 45 processes were under enriched and mainly comprised of cellular components (cellular, organelle part) along with translation, metabolic process (carbohydrate, peptide, nucleotide & coenzyme) with values less than 1 %. Purine, ATP, organ nitrogen compound biosynthetic & other metabolic process GO terms with a percentage ranging from (2-10 %) were moderately under enriched.

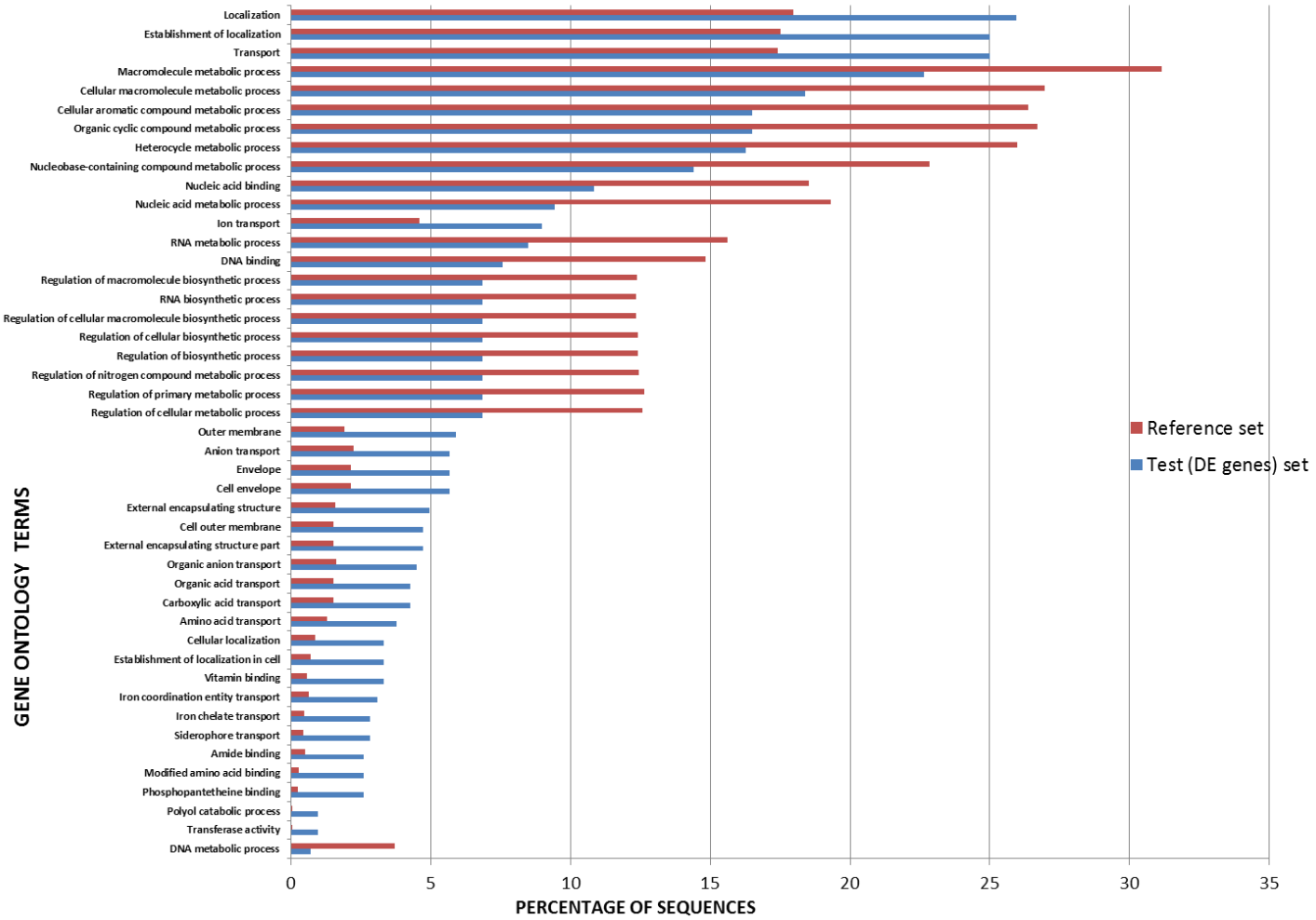


Figure 5.12: GO term enrichment analysis of *P. poae* genes upregulated during pathogenesis. The figure represents all the significant GO-term categories found significantly enriched compared to the reference set (all genes present on the RNA-Seq data) after a Fisher's exact test and Benjamini and Hochberg multiple testing correction (FDR,0.05). The test set percentage indicates the percentage of up regulated genes belonging to a GO term category compared to all up-regulated genes used in the GO-term analysis while the reference set percentage indicates the percentage of a particular GO-term category compared to all genes with GO-terms on the RNA-Seq data.

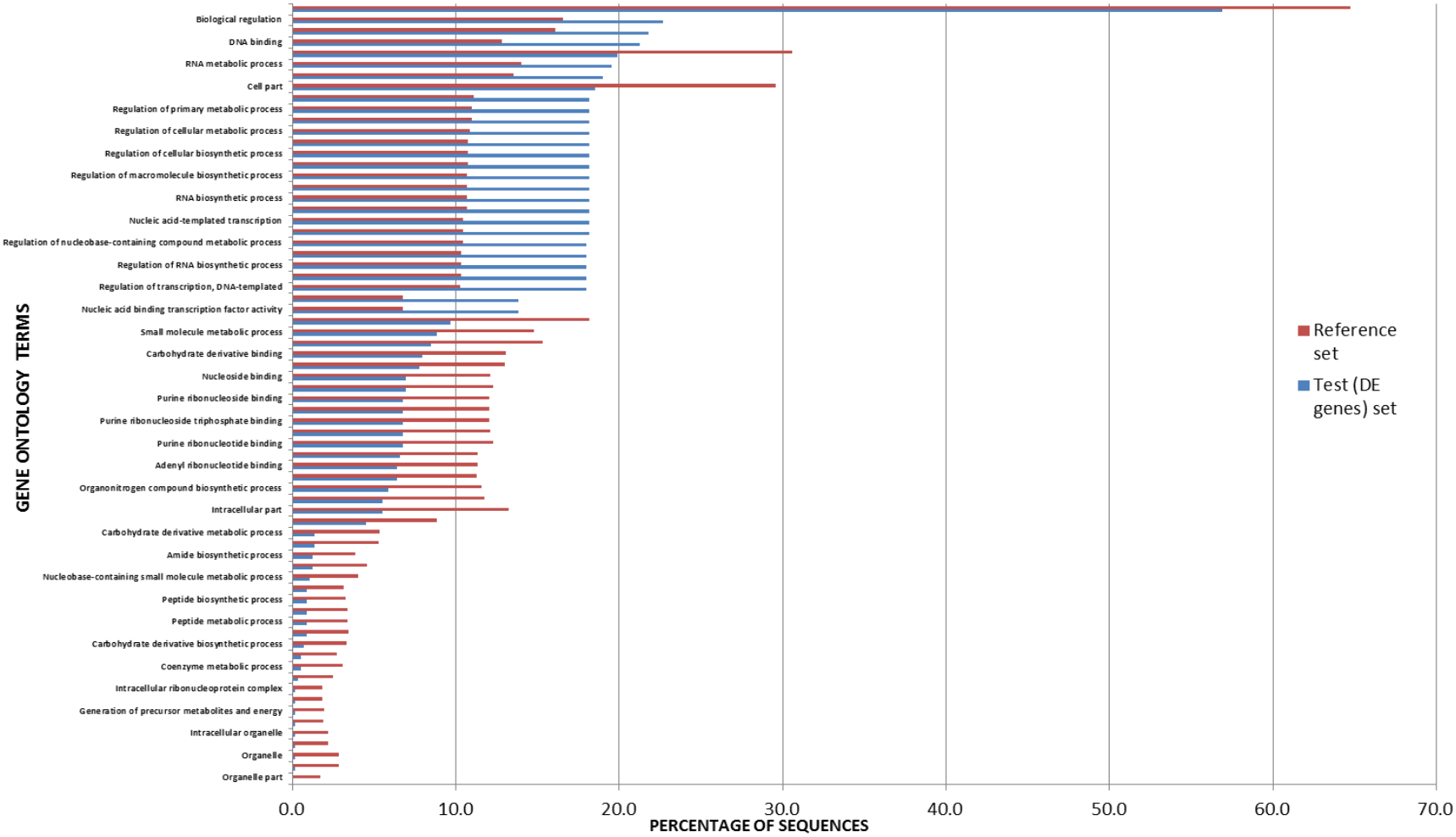


Figure 5.13: GO term enrichment analysis of *P. poae* genes down regulated during pathogenesis. The figure represents all the significant GO-term categories found significantly enriched compared to the reference set (all genes present on the RNA-Seq data) after a Fisher's exact test and Benjamini and Hochberg multiple testing correction (FDR,0.05). The test set percentage indicates the percentage of down regulated genes belonging to a GO term category compared to all down regulated genes used in the GO-term analysis while the reference set percentage indicates the percentage of a particular GO-term category compared to all genes with GO-terms on the RNA-Seq data.

5.7.7 Validation of RNA Seq experiment by qRT PCR

To validate the RNA-Seq analysis data, 10 genes from each aphid and bacteria differential expressed gene set were selected (Table 5.5) for confirmation by QPCR. The selection of genes to use with QPCR was considered based on major altered host and bacterial gene profiles, which showed less variation between FPKM values between replicates. Another major consideration was to avoid any multiple copy genes which might cause large variation in quantifying the expression. In the case of the eukaryotic genes, which are a set of alternatively spliced transcripts, an appropriate transcript was selected from transcript differential FPKM values. This particular transcript sequence was considered for primer designing of the same altered gene. The altered bacterial profile showed major events which allow them to colonise and cope with harsh condition inside the aphid guts. These genes consisted of those associated with iron limitation (iron uptake and transport genes), low redox stress (antioxidant genes), metabolism, transport and other process (motility and DNA recombination). The aphid transcriptomic data showed limited expression of gut specific defensive genes such as proteases and other detoxifying genes. All these genes were quantified for their transcript levels in qRT-PCR on the same replicate of RNA samples which were sent for RNA sequencing.

In all biological replicates, 20 genes showed concordant changes between the RNA-Seq data and qRT-PCR data. The Pearson coefficient was calculated between two methods and R^2 ~ 0.99 suggests that the RNA-Seq data are highly accurate and robust (Figure 5.14).

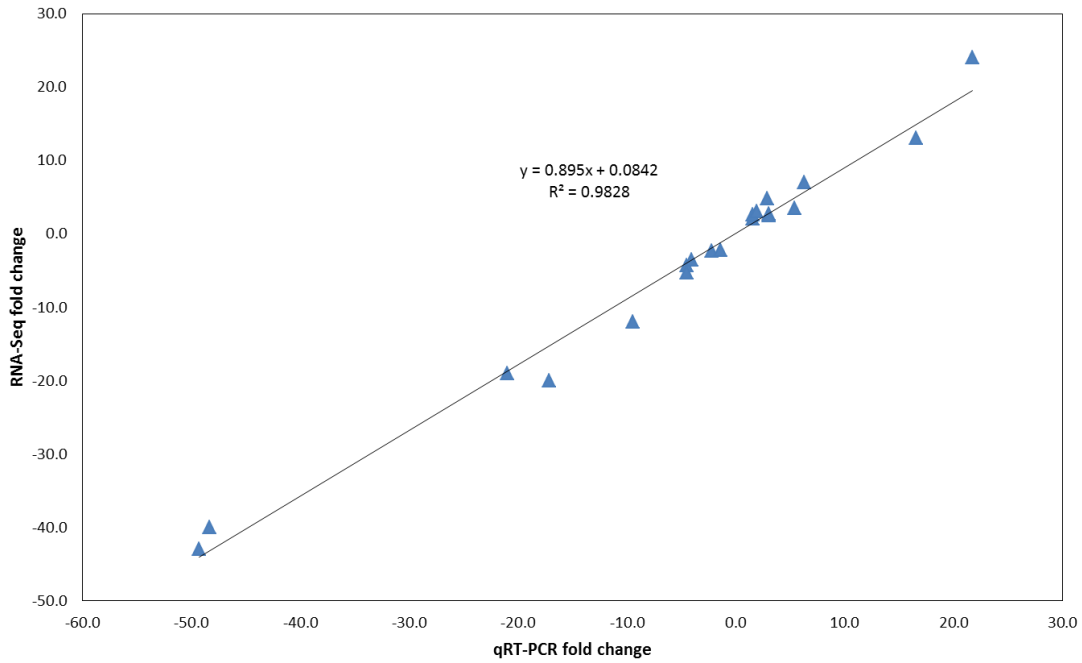


Figure 5.14: qRT-PCR validation of RNA-Seq results. Validation of gene expression (20 genes) using Pearson's correlation (r) between fold changes observed in qRT-PCR and RNA-Seq results.

Table 5.5: qRT-PCR validation of RNA-Seq data.			
Gene (Gene_Id)	**Process**	**QPCR (fold change)**	**RNAseq (fold change)**
Bacteria QPCR Gene sets			
Thymine DNA-glycosylase (CDH05_24650)	DNA repair	-49.3	-43
AHYP -Alkyl hydroperoxide reductase (CDH05_15375)	Oxidative stress	-21	-19
RND efflux membrane fusion protein (CDH05_27355)	Membrane transport	-17.2	-20
Fimbriae usher protein StfC (CDH05_20275)	Motility	-9.5	-12
Arginine deaminase (CDH05_16445)	Amino acid metabolism	-4.5	-5.3
Hypothetical protein-toxin (CDH05_22015)	transcription promoter (toxin)	2.9	4.8
ArapX -Serine protease (CDH05_16530)	Protease	3	2.5
PvdD-NRPS (CDH05_05885)	Iron uptake process	6.3	7
PvdF-synthetase (Pyoverdine biosynthesis) (CDH05_05870)	Iron uptake process	16.5	13
EfeoB1 (peroxidase) (CDH05_24265)	Iron transport	21.7	24
Aphid QPCR Gene sets			
Carotenoid desaturase (MYZPE13164_G006_v1.0_000134430)	Cholesterol Metabolism	-48.35	-40
Facilitated trehalose transporter (MYZPE13164_G006_v1.0_000108000**)**	Membrane transport	-4.56	-4.3
Olfactory receptor (MYZPE13164_G006_v1.0_000058010**)**	Behaviour	-4.1	-3.5
Major facilitator superfamily (MYZPE13164_G006_v1.0_000166060**)**	Membrane transport	-2.21	-2.35
Lycopene cyclase (MYZPE13164_G006_v1.0_000134390**)**	Cholesterol Metabolism	-1.45	-2.18
Noggin (MYZPE13164_G006_v1.0_000056300**)**	Growth regulator	1.5	2.1
Cyc-P450 (MYZPE13164_G006_v1.0_000083350**)**	Detoxification	1.54	2.6
Cuticular protein (MYZPE13164_G006_v1.0_000169000**)**	Detoxification	1.88	3
Venom protease (MYZPE13164_G006_v1.0_000046800**)**	Detoxification	2.99	2.7
Cathepsin-B (MYZPE13164_G006_v1.0_000049160**)**	Detoxification	5.37	3.5
Table depicts fold changes in gene expression of selected 20 candidate genes between control and treatments as assessed by RNA-Seq and qPCR.			

5.8 Discussion

Understanding the factors enabling infection of aphids by bacterial pathogens is critical to advancing effective aphid biocontrol strategies. *Pseudomonas poae* is an example of a highly pathogenic bacterium to aphids. Simultaneous analysis of the transcriptional response of the host (aphid) and the pathogen (bacteria) during infection is one route to enhance our understanding of the major cellular and metabolic changes of the host and pathogen that underlie pathogenicity and a compatible interaction.

My initial attempts to carry out a dual RNA-Seq analysis of the host and pathogen transcriptomic response during infection were unsuccessful. This was largely due to a failure to obtain sufficient sequence coverage of bacteria when inside the aphid host, where it makes up a very small percentage of the total RNA extracted. By carrying out a time course experiment of bacterial infection of aphids using qPCR I was able to determine the optimum time to sample in order to obtain the highest possible bacterial load. This approach along with employing a bacterial enrichment methodology, to increase the representation of bacterial transcripts in the infected aphid sample, allowed me to be much more successful in a follow on RNA-Seq experiment.

This approach generated enough reads to get good coverage of aphid and bacterial transcripts against their respective genome. Despite the measures mentioned above the RNA sequencing of aphids treated with bacteria revealed just 1 % of the reads obtained mapped to bacteria. To avoid low read bias, I selected a minimum cut-off of FPKM value of 5 for further analysis. Additionally, quality control Csdensity and box plots showed equal distribution of FPKM across all replicates of aphid and bacteria suggesting our methodology and subsequent analysis steps were sound.

The key results from transcriptome profiling of aphids under bacterial infection are discussed below, initially focusing on the aphid response then the bacteria.

5.8.1 Aphid transcriptome

5.8.1.1 Dynamics of salivary gland and gut specific defence mechanisms inside aphids

The whitefly gut study demonstrated detoxification-related genes such as cytochrome P450s, GSTs and glucuronosyltransferases were also found specifically expressed in the guts. Other digestive genes like proteases, lipases, esterases, and alpha-glucosidase along with 'Facilitated trehalose transporter' and 'MFS transporter' which are involved in removal of toxics & foreign material are majorly reported in the gut and salivary gland (Xia *et al.,* 2013).

In this study, induction of salivary gland and gut specific digestive enzymes including leucyl-cystinyl aminopeptidase, metalloproteases, lipases and mucin-like suggested active digestion of aphids on *P. poae* treated diet (Richards *et al.,* 2010; Huang *et al.,* 2016) (Appendix Table 3B). The over enriched gene ontology (GO) process serine and cysteine endopeptidase activity also indicated differential expression of proteases which may be a response to the bacteria inhibitors released during infection. However, modified protease activity of *M. persicae* may have adverse effects as it can be detrimental to critical gut structures, in addition to the potential damage caused by bacteria toxins released as a defence mechanism (Kutsukake *et al.,*2004) . Thus, in order to protect itself from internal and external proteinases, it is possible that *M. persicae* differentially regulates protease as observed in this study (Table 3 C). Moreover, elevation of two apoptotic proteins of *M. persicae* salivary gland which were stimulated by rho protein signal activity and also complemented by increase transcription of metalloproteases, serine and cysteine proteases is similar to reports of autophagy cell death in Drosophila (Baehrecke, 2003) (Appendix Table 3A). The major gut specific genes glucosidase, aminopeptidase N-like protein, facilitated trehalose transporter and solute carrier family 46 member 3-like were also expressed during feeding (Cristofoletti *et al.,* 2006; Ye *et al.,* 2014). Impairment of major facilitator super domain and solute carrier family transporters slow down absorption of amino acids, sugar and lipids in the midgut epithelium (Appendix Table 3D). Trehalose transporter proteins, involved in pathogen and drought response and arthropod virulence to toxins, named aminopeptidases were also downregulated (Appendix Table 3B). These results lend support to the hypothesis that after ingesting bacteria*, M. persicae* employs a suite of saliva and midgut defensive countermeasures to overcome resistance from bacteria.

5.8.1.2 Transcription of other cellular process led to pathogen invasion in aphids

In the current study, differential expression of *M. persicae* genes involved in cell-cell interaction, endocytosis, vesicle trafficking, and the cytoskeleton was observed inside treated aphids. The variance of these genes may indicate that changes in cytoskeletal arrangement, cell–cell interaction and membrane trafficking were induced by bacteria to facilitate its colonisation within the host insect, or these cellular modifications as part of a host defence response against the bacterial replication.

In a previous study, the involvement of *Ph. luminescens* TT01 type III secretion system (T3SS) in host cell invasion was observed, where effector proteins were injected into host cells in the early stage of infection. Entry of these effector proteins modulate the host endocytic system, which led to internalization of bacteria (Brugirard-Ricaud *et al.,* 2005) . In the current

study, some type III secretion system genes showed downregulation of expression indicating they were unlikely being used at this stage of infection (Appendix Table 5H). It might be hypothesized that invasion of insect cells by the bacterium possibly occur by Type III secretion genes, which was described in a *Galleria mellonella* caterpillar model by *P. aeruginosa* PA14. Another study characterized the role of *P. taiwanensis tccC* toxin in insect pathogenicity and invasion of bacterial cells in the gut of *Plutella xylostella* induced host programmed cell death-related genes (JNK-2 and caspase-3) that led to cell death.

It is possible that *P. poae* exploits conserved host cellular machinery within the insect to facilitate its infection, and the above-mentioned gene changes may be induced by *P. poae* virulence factors as part of the pathogen's cellular invasion strategy (Appendix Table 3C). Alternatively, *M. persicae* may be differentially regulating genes which involved in cellular adhesion and endocytosis in an effort to prevent *P. poae* from takeover these systems for its own advantage (Appendix Table 3C). Intracellular pathogens have been accounted to hijack the host ubiquitin system and manipulate the host actin cytoskeleton for successful invasion (Mortimer, 2011). Upregulation of ubiquitin proteins may reflect manipulation of the host (*M. persicae)* proteolysis system to target the pathogen protein for degradation (Appendix Table 3C).

5.8.1.3 Transcription of aphid immune and stress related proteins

Many previous studies have suggested an important role of insect epithelial barriers and conserved toll receptors in recognition of pathogens which trigger signalling cascades that direct expression of a battery of antimicrobial peptides, cytokines, and other immune mediators (Govind, 2008; Laughton *et al.,* 2011).

Similarly in the altered aphid response observed in the current study, "uncharacterized family 31 glucosidase KIAA1161-like" gene with predicted glucosidase activity may play a role against Gram-bacteria recognition. Up-regulation of stress related genes such as 'cuticular', 'takeout, sterile alpha and TIR motif- related (SARM) and leucine rich repeat domain, in response to infection is consistent with an insect stress response (Marmaras *et al.,* 1993; Pal *et al.,* 2008; Waterhouse *et al.,* 2010; Hou *et al.,* 2013) (Appendix Table 3A). However, other responses such as no expression of immune signalling pathways and downregulation of lysozyme and drug metabolism genes suggest that the *M. persicae* defence responses may be either not activated or suppressed, enabling colonisation of the pathogen through the insect (Appendix Table 4A,B). Similar to other Hemiptera, the repertoire of antimicrobial defence mechanisms is reduced in aphids relative to other insects (Gerardo *et al.,* 2010). The requirement to accommodate their colonization by beneficial symbionts has been appealed

to explain the lack of the Immune Deficiency (IMD) pathway and antimicrobial peptide genes in hemipteran genomes (Gerardo *et al.,* 2010).

5.8.1.4 Alteration of aphid metabolism

The present study revealed modulation in metabolic activity of infected aphids, and this may be the result of behavioural and physiological responses to bacterial infection. Increase in proline biosynthesis activity indicate a potential role in protection against increased cellular stress or mechanical injury (e.g. tissue disruption, cell death) in response to infection with nematodes and their bacteria (Krishnan *et al.,* 2011) . The induction in breakdown of triglycerides (lipase activity) and metabolism of the breakdown products into compounds which can enter glycolysis or the citric acid cycle for energy production related to energy storage and utilization are associated with feeding on the bacterial pathogen (Appendix Table 3E). The coordinated downregulation of glutathione metabolic enzymes such as Gamma-glutamyltranspeptidase and omega-amidase which are involved in antioxidant and detoxification suggest suppression of aphid immune responses (Appendix Table 3E). Major carotenoid biosynthetic genes which are responsible for parasitism and predation may be the result of behavioural changes induced by bacteria infection (Cazzonelli, 2011) (Appendix Table 3E).

Moreover, up-regulation of the *M. persicae* flight muscle protein titin and downregulation of olfactory receptor activity suggests that changes in insect physiology potentially affect responses to visual and olfactory cues in physiological and nutritional changes during infection. Behavioural studies are needed to determine whether the changes in metabolic enzymes observed in infected aphids have an impact on host plant choice, feeding patterns, and flight characteristics.

5.8.2 Bacteria transcriptome

5.8.2.1 Iron limitation increased transcription of iron acquisition systems in *P. poae*

Iron is essential for most invading microorganisms during the course of an infection, and both animals and plants have evolved elaborate immune strategies to limit iron availability to microorganisms (De Gregorio *et al.,* 2001; Irving *et al.,* 2001; Nappi & Vass, 2000). Like other organisms, insects have evolved distinctive forms of the serum iron transport protein, transferrin, and the storage protein, ferritin (Nichol *et al.,* 2002). Ferritin is evenly distributed among the haemolymph and abundant in posterior regions of the midgut. At an aerobic stage, under oxidative conditions, iron (in the form of Fe^{3+}) is present, which at pH 7 is

completely insoluble. The host fulfils its own iron requirement by synthesizing the proteins transferrin and ferritin, which very tightly bind iron (Yeom, Imlay, & Park, 2010). To cope with iron deprivation, under these conditions, bacteria are highly inventive and evolved a number of intricate mechanisms to fulfil their iron requirements. Although some pathogens, like Neisseria, are able to take-up iron directly from transferrin, this is not an option for *P. aeruginosa* (Cornelissen, 2003; Noinaj *et al.,* 2012). *P. aeruginosa* can use different strategies to acquire iron such as production of siderophore (pyoverdine and pyochelin) and the uptake of ferrisiderophores via TonB-dependent receptors (TBDR), the uptake of xenosiderophores (not produced by the bacterium itself) and uptake of the haem molecule from the host hemoproteins.

In the current study, higher expression of iron acquisition systems in treated *P. poae*, such as the Pyoverdine biosynthesis gene and Siderophore uptake iron transport, are upregulated in order to survive in the iron limiting conditions (Appendix Table 5A). Besides, the upregulation of haem acquisition system genes, which are involved in haem degradation to release the bound iron, provides another line of evidence that bacteria actively respond to the iron depleted conditions inside aphids (Lim *et al.,* 2012) (Appendix Table 5A). In addition to Siderophore iron uptake, other iron transport systems such as EfeUOB, metal transporters (Zn), ABC transport systems, that are predicted to be involved in iron (III) uptake across the cytoplasmic membrane, were highly transcribed under the iron limiting condition in this current study (Lim *et al.,* 2012) (Appendix Table 5A).

These current findings are consistent to upregulation of iron acqusistion, uptake and transport in iron limiting studies of *P. fluorescens* Pf-5, *P. aeruginosa* and *P. syringae* (Ochsner *et al.,* 2002; Palma *et al.,* 2003; Bronstein *et al.,* 2008; Lim *et al.,* 2012)

5.8.2.2 Oxidative stress response and iron storage

All aerobic bacteria generate toxic oxygen derivatives {superoxide (O_2) and hydroxyl radicals (HO)} formed due to uncontrolled electron transfers. These radicals further react with iron to produce highly reactive and damaging hydroxyl radicals through the Fenton reaction {iron (II) + H_2O_2 R iron (III) + HO} ((Huang *et al.,* 2002) Hence, bacteria have developed strategies to meet the physiological requirement of iron uptake and minimize damage caused by iron induced reactive oxygen species.

Bacteria have employed various antioxidant enzymes (superoxide dismutase [SOD], catalase, and peroxidase), DNA repair enzymes and DNA binding proteins (e.g., Dps -DNA binding

protein) and free-radical-scavenging agents to cope with reactive oxygen species (Cornelis *et al.,* 2011). The excess cellular iron (II) is converted to iron (III) through ferroxidase activity imparted by bacterioferritin and stored as a ferric (Fe^{3+}) mineral within bacterioferritin.Some studies reported differential expression of bacterioferritin because the need for iron storage was reduced under low-iron conditions, such as downregulation of bacterioferritin in *P. syringae,* and bacterioferritin-associated ferredoxin gene up-regulation in *P. aeruginosa* and *P. syringae* (Ochsner *et al.,* 2002; Palma *et al.,* 2003; Bronstein *et al.,* 2008;; Yao *et al.,* 2011).

Similar findings were observed in my study, bacterioferritin gene, encoding a non-haem binding bacterial ferritin, was down-regulated and the bacterioferritin-associated ferredoxin gene was upregulated in treated bacteria suggesting another mechanism to compensate for iron deprived conditions (Appendix Table 5D).

Another example of reduction of iron demand was differential expression of two different forms of superoxide dismutases; one utilizes iron as cofactor (*sodB*), which is down-regulated in iron-limited conditions, presumably due to the reduced availability of iron in the cells. Conversely, a manganese-based superoxide dismutase (s*odA*) was up-regulated (Polack *et al.*, 1996; Hassett *et al.,* 1997). The coordination of bacterial responses to iron limitation and the defence against oxidative stress has been proposed previously (Touati, 2000).

Similarly, my study describes the upregulation of a manganese-based superoxide dismutase (s*odA*) with down regulation of iron superoxide dismutase (*sodB*), suggesting reduced expression of iron cofactor-dependent enzyme, when iron is in short supply. Additionally, coordinated downregulation of catalase and alkyl hydrogen peroxide strongly suggest that *P. poae* adapts its repertoire of oxidative stress response enzymes by limiting their expression under low redox stress (Appendix Table 5D).

5.8.2.3 Iron limitation affects transcription of other process in *P. poae*

Many studies have demonstrated that iron limiting conditions lead to changes in gene expression profiles involved in processes which require iron cofactors or are linked with transcriptional factors of iron homoeostasis. The oxygen Stress regulator “OxyR” co-ordinately regulated oxidative stress genes (*katB, ahpB, or ahpCF*) and DNA repair genes upon availability of iron. In low iron demand, expression of DNA repair and oxidative stress genes were repressed in *P. aeruginosa* (Ochsner *et al.,*2000). The microarray data of *P. aeruginosa* showed differential expression of multiple terminal oxidases for aerobic respiration under iron and other stress conditions (Ochsner *et al.,* 2002; Kawakami *et al.,* 2010). Another study in *P. aeruginosa* suggested sulphur assimilation by taurine and

alkanesulphones transporters are also linked with iron homeostasis and downregulation of these genes reduced demand for iron in the cell (Amich *et al.,* 2013) (Appendix Table 5H).

My data revealed similar altered profiles in response to low iron. The transcription of genes encoding subunits of a cytochrome o ubiquinol oxidase increased and subunits of cbb3-type cytochrome c oxidases decreased under iron limitation suggesting iron availability alters the preferred branch of the electron chain utilized by *P. poae* growing in an aerobic environment, providing a mechanism for the bacterium to conserve iron demands on the cell (Poole & Cook 2000; Kawakami *et al.,* 2010). (Appendix Table 5G).

The previous study identified the *oxyR-recG* locus in *P. aeruginosa*, which is a genetic link between an oxidative stress gene and a DNA repair gene. They described that the oxygen Stress regulator "OxyR" co-ordinately regulates antioxidant genes (*katB, ahpB, or ahpCF*) and acts as first line of defence against oxidative stress inside host tissues. Another strategy to survive these harsh conditions is to maintain an efficient DNA repair system (Ochsner *et al.,*2000).

In this study, repression of *P. poae* antioxidant genes (*katB, ahpB, or ahpCF*) and DNA repair genes suggested that low redox conditions exist inside aphid tissues (Appendix Table 5D). These findings conflict with previous studies of *P. taiwanensis* which described induction of antioxidant gene expression inside the *Drosophila* gut. Besides these, no study has demonstrated expression of DNA repair genes inside the insect gut during bacterial pathogenesis. However, the low ROS level inside aphids reduced the chances of damage to bacterial DNA, which likely accounts for repression of *P. poae* DNA repair genes (Appendix Table 5G).

These findings also proposed that infected aphids did not generate oxidative stress against *P. poae* colonisation.

5.8.2.4 Transcription of bacteria flagella and adhesion proteins

Iron homoeostasis is also associated with transcription of the flagellar gene biosynthesis and chemotaxis. The previous reports indicated motility of *P. aeruginosa*, *P. syringae* and *P. putida* is promoted by iron limitation (Taguchi *et al.,* 2010; Matilla *et al.*, 2007; Deziel *et al.,* 2003; Singh *et al.,* 2002) . It was suggested that this phenomenon is a chemotactic response of *P. aeruginosa*, allowing it to migrate to another location in search of nutrients (Mulligan & Gibbs 1989; Deziel *et al.,* 2003). Studies have also demonstrated that ferritin coupled with low iron conditions stimulates twitching, a specialized form of surface motility, which caused bacteria to move across the surface instead of producing cell clusters and biofilms in *P.*

aeruginosa (Singh *et al.,* 2002; Patriquin *et al.*, 2008). The higher expression of few flagellar genes and chemotaxis protein along with coordinated reduced expression of biogenesis of fimbriae and Type IV pilus biogenesis protein in this study likely reflects the role of iron in modulating *P. poae* adhesion activity in a similar way (Appendix Table 5E).

5.8.2.5 Defensive mechanisms

Besides iron starvation, bacteria will have faced variations in pH conditions across the insect gut and haemocoel. The pH of haemocoel & foregut is usually 5-6, the anterior part of midgut 5.5 -6 and the rest of the part of gut pH varies from 7.5-8.5 (Cristofoletti *et al.,* 2003). Some studies revealed different strategies of pathogens to survive harsh conditions and lead to invasion of the host cells. To survive in low pH conditions of the gut region, *Helicobacter* species secreted urease enzyme for colonization (Belzer *et al.,* 2007). Additionally *P. entomophila* secreted proteases like AprX which cause haemolymph bleeding and anaphylactic responses during invasion in *Drosophila* and silkworm (Liehl *et al.,* 2006).

In this study, coordinated upregulation of urease gene and ammonia transporter activity to efflux extra amount of ammonia from the cytoplasm is a proposed explanation of survival of *P. poae* inside the aphid gut (Heermann & Fuchs, 2008) (Appendix Table 5D).Higher expression of various proteases such as Aminopeptidase N, putative cysteine proteases and alkaline protease AprX (serralysin) are similar to findings reported in the study on haemolymph bleeding in silkworm (Ishii *et al.,* 2014) (Appendix Table 5D).

Another level of defence, membrane Resistance Nodulation cell Division (RND), drug & multidrug metabolite efflux transporters are responsible for export of antibiotics, drug and other toxic compounds across the membrane. In the current study, downregulation of these transporters may be resulting from no antimicrobial secretion from aphids or they are suppressed by bacteria pathogenesis (Appendix Table 5C). Our findings conflict with previous work, which observed increased expression of efflux protein gene expression inside the pea aphid after attack of *Dickeya dadantii* (Costechareyre *et al.,* 2013).

The strategies detailed above suggest bacteria can survive in unfavourable conditions of host and secrete many proteins which facilitate their colonisation inside the aphids.

5.8.2.6 Modification in bacteria metabolism for survival inside aphids

In this study, overexpression of various bacteria metabolic enzymes associated with lipoprotein, lipopolysaccharides, fatty acids, chorismate (Siderophore) biosynthetic processes may be a result of physiological changes to survive under extreme conditions inside host cells. The major constraint on bacteria is to conserve iron sulphur assembly

enzymes in low iron conditions, all enzymes with Fe-S clusters in their catalytic cores, many of them in the tricarboxylic acid cycle (TCA), were downregulated (Appendix Table 5B). Inositol catabolism enzymes are upregulated in treated *P. poae* which suggest that this sugar alcohol is used as a carbon source and end product of acetyl-coenzyme to enter into the TCA cycle (Appendix Table 5B). The upregulation of Glyoxlate genes named "Isocitrate lyase" and "Malate synthase G" which allows growth on C2 compounds by bypassing the CO_2-generating steps of the TCA cycle during pathogenesis inside host cells was supported by similar studies in *P. aeruginosa* (Dunn *et al.,* 2009). Finally, end product of glyoxylate cycle malate and oxaloacetate enter into the gluconeogenesis cycle. The over enriched "phosphopantetheine binding" GO terms associated with both fatty acid and Siderophore metabolism suggest another coordinated metabolic response of bacteria to cope with stress inside aphids (Appendix Table 5B).

However, the under-enriched GO term associated with regulators of metabolic process indicated downregulation of various amino acids (arginine, valine, Isoleucine, Proline and other amino acids) and fatty acid degradation suggestive of conserved energy metabolism for *P. poae* survival inside aphids (Appendix Table 5B).

In summary, the first RNA Seq did not provide much information of altered gene profiles of both bacteria and aphid. Nevertheless, lower expression of aphid stress (Cytochrome p450 & heat shock) and other cell morphogenesis genes at low infective dose suggested that these potential genes could be utilised further as key targets in early infection of *P. poae* (Appendix 1). In future, a comparative account of these target gene profiles at two different doses through quantitative QPCR method would help in understanding how bacterial load could play a role in suppression of aphid defence related genes and pathogenesis. Moreover, expression profiles of control bacteria transcripts when growing in Mittler diet at two different doses and at different time points would help to understand the physiological adaptation of bacteria, including nutrition, metabolism, transport and regulation in sucrose rich medium (Appendix Table 1 & 5).

Although the MICROBEnrich procedure allowed enrichment of bacterial transcripts from infected aphids, the late time point for gene profiling resulted in loss of a few early expressed genes such as major bacterial toxin and effectors, which could be involved in suppression of host immune genes. Similarly, the molecular mechanisms of aphid defence and immune related genes in early infection steps were not evaluated.

Taken together, transcriptome profiling through RNA-Seq provides a commendable approach for the precise assessment of transcript levels and transcript isoforms in the host pathogen infection model.

RNA-Seq analysis of *Myzus persicae* infected by *P. poae* bacteria reveals transcriptional changes in the regulation of 193 genes in aphids and 1325 genes in bacteria, many of which have not been shown previously to participate in immune processes against pathogenic infections. Furthermore, it is demonstrated that not only stress/virulence - related genes but also genes involved in metabolic processes are modulated during pathogen infection. The results obtained could benefit future in-depth studies on the role of candidate genes as potential targets for insect control, and improve our understanding of host-pathogen interactions.

6 Investigation of virulence factors in *Pseudomonas poae*

6.1 Introduction

Insects are the most diverse animal species that cause plant damage by directly feeding on above-ground and below-ground plant parts. The use of chemical pesticides is restricted due to environmental risks, concerns for public health and the rapid development of resistance in target pest. Entomopathogenic bacteria, such as *Bacillus thuringiensis* (Bt) and *Photorhabdus/Xenorhabdus* species, are promising candidates to kill insects with no harmful effects on humans. However, *Bacillus* shows less environmental persistence due to its sensitivity towards solar irradiation as well as to the chemical environment on plant leaves, and it is not a competitive plant colonizer (Bizzarri & Bishop, 2008; Raymond *et al.,* 2010). *Photorhabdus* and *Xenorhabdu*s species require a nematode vector to release their toxins into the insect haemocoel, which brings too much complexity in its formulation and application, especially for soil-borne pathogens (Kupferschmied *et al.,* 2013). Certain bacteria of the genus *Pseudomonas* could constitute a promising alternative to the above groups of commercialized entomopathogens in addressing the major problem of phytophagous and soil dwelling pests (Kupferschmied *et al.,* 2013).

The plant beneficial fluorescent *pseudomonas* is a highly diverse group which can inhabit a wide range of environmental niches. Many root-colonizing members of the *Pseudomonas* group have been studied for their ability to suppress root diseases, to promote plant growth and to induce systemic resistance. They display an array of secondary metabolites with potent antifungal activity to inhibit pathogen growth through direct antibiosis. The metabolites such as phenazines, 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, hydrogen cyanide (HCN), and cyclic-lipopeptides have demonstrated their role in plant disease suppression (Haas & Keel, 2003; Raaijmakers *et al.,* 2010).

Gene loci similar to *Photorhabdus Tc* genes have been reported in the insect-associated *P. entomophila* and other non-insect-associated *Pseudomonas* species. *P. entomophila*, the first strain found to be pathogenic to *D. melanogaster,* triggered a systemic immune response and displayed an ability to orally infect and kill both larvae and adult of the insect (Vodovar *et al.,* 2005). Three TccC-type toxins and one TcdB-type Tc toxin were found in *P. entomophila* genome which most likely play a major role in the pathogenicity of *P. entomophila*, as TccC and TcdB proteins have insecticidal activity (Vodovar *et al.,* 2006).

Some other *Pseudomonas* spp., such as the leaf pathogen *P. syringae* pv. tomato and the soil saprophyte *P. fluorescens* Pf0-1, have no known association with insects, but their genomes also encode Tc toxins (Buell *et al.*, 2003).

The recent genome sequencing of *P. protegens* Pf-5 revealed features that contributes to its commensal lifestyle on plants such as iron acquisition and stress tolerance. Additionally, the bacterium not only harbours antifungal metabolite synthesis genes, but it possesses the potent insect toxin Mcf1 of the entomopathogen *P. luminescens* (Paulsen *et al.*, 2005). This is the first identification and analysis of a proteinaceous insect toxin from plant-associated pseudomonads. The insect toxin Mcf (Makes caterpillars' floppy) orthologue has been found in *P. fluorescens* group strains Pf-5 and CHA0 as part of an eight-gene cluster which has been designated as "fit" for *P. fluorescens* Insecticidal Toxin. The gene *fitD* codes for the actual toxin which has a molecular weight of 327 kDa. *fitD* is flanked by four genes (*fitABC-E*) predicted to encode a type I secretion system and three genes (*fitFGH*) coding for putative regulatory proteins (Péchy-Tarr *et al.,* 2008). These strains were lethal to larvae of *M. sexta* and the greater waxmoth *Galleria mellonella* upon injection of very low doses into the haemocoel of these insects. Another plant associated bacterial strain *P. chlororaphis* PCL1391 also harbours the Fit gene cluster and kills insects via oral infection.

Spraying plant leaves with low doses of these bacterial suspensions efficiently killed several agriculturally important lepidopteran pest insects, notably *D. melanogaster*, the African cotton leafworm *Spodoptera littoralis*, the tobacco bud worm *Heliothis virescens*, and the diamondback moth *Plutella xylostella* (Ruffner *et al*., 2013). However, FitD mutants still exhibited a considerable amount of insecticidal activity which suggests additional virulence factors are present that still need to be explored.

In support of this finding, this study has indicated that some plant derived bacteria were pathogenic to different species of aphid (Livermore, 2016). The ability of *P. poae* to orally infect and kill different kinds of aphid insecticide clones makes it a promising model for the study of host-pathogen interactions and for the development of biocontrol agents against insect pests. Additionally, *P. poae* was successful in colonising different plant species and showed excellent efficacy to control aphid infestation. To characterise the interaction between aphid and bacteria during pathogenesis, RNA Seq was used to analyse altered gene profiles and identify potential bacterial virulence factors which are involved in killing mechanisms.

The next step is to characterise the *P. poae* genome sequence and explore genetic elements associated with insecticidal and other biocontrol capabilities. Combining the results of the

RNA-Seq with extensive genome characterization data is helpful to elucidate the killing mechanism of aphids.

My aims are as follows:

1. To characterise the *P. poae* genome and relate to other *Pseudomonas* species.
2. Functional characterization of *P. poae* genome
3. Identification of virulence factors by knock-out mutagenesis

6.2 Bio-informatics analysis

6.2.1 Genome assembly

P. poae was sequenced by a service provider (University of Exeter) using the Illumina HiSeq platform (Illumina). The *de novo* assembly was prepared by sequencing service through use of Velvet assembler tool according to the user's manual (Zerbino, 2011). After assembly, 217 contigs were generated which were further used for functional analysis.

6.2.2 Functional annotation

All fasta sequences were loaded into Blast2GO and BlastX analyses were performed to search *P. poae* nucleotide sequences against the nr protein database using an e-value cut-off of 10^{-3} and reporting a maximum of 20 'hit' sequences per query. Next, mapping was done to retrieve GO terms associated to the hits obtained after a BLAST search. Additional steps in mapping were performed to retrieve UniProt IDs making use of a mapping file from PIR (Non-redundant Reference Protein Database) including PSD, UniProt, SwissProt, TrEMBL, RefSeq, GenPept and PDB. After mapping, the search results were finally subjected to function Annotation > Perform Annotation Step menu to perform GO annotation with default parameters. Additionally, the KEGG map module allows the display of enzymatic functions in the context of the metabolic pathways in which they participate. The EC codes are highlighted with different colours (one colour for each EC) in the pathway map.

6.2.3 Comparative genome analysis

Evolutionary relationships between *P. poae* strain PpR247 and their closest genetically related species were investigated using the Multilocus sequence typing (MLST) approach developed by Andreani *et al.,* (2014) to characterize the *P. fluorescens* group. The seven MLST loci sequences *glnS, gyrB, ileS, nuoD, recA, rpoB, and rpoD* from 97 strains (Andreani *et al.,* 2015) were downloaded from NCBI, while those from genomes sequenced in this study were extracted blasting the MLST sequences of the reference genome *P. fluorescens* A506

against the genomes. This dataset was enriched with the MLST sequences extracted from the 79 genomes of the *P. fluorescens* species and most related species gathered in the genetic cluster 2 (Monteil *et al.,* 2014) in which all loci were detected (using a BLAST word size of 11 pb, a minimum sequence identity of 70 % and alignment length of 50 %). Gene sequences were aligned independently using MUSCLE and then concatenated into a single alignment of 3541pb among which 1428 sites were polymorphic. A maximum-likelihood (ML) tree was built with RAxML 8.2.6 under the GAMMA model of rate heterogeneity using empirical nucleotide frequencies and the GTR nucleotide substitution model. A total of 249 bootstrap replicates automatically determined by the MRE-based bootstrapping criterion were conducted under the rapid bootstrapping algorithm, among which 100 were sampled to generate proportional support values. All this work was performed at different lab by Caroline Monteil due to availability of appropriate bioinformatics resources (such as *Pseudomonas* genome database & tools).

To describe the *P. poae relationship* within *P. fluorescens* group, I focused on the 22 strains within the *Pseudomonas* group of which the complete genome or draft genome sequence is available. Additionally, *P. syringae* pv. tomato str. DC3000 was included as the outgroup. Phylogenetic relationships among the selected 24 sequenced *Pseudomonas* species were investigated by generating phylogenetic trees using concatenated alignments of 4 highly conserved housekeeping genes: 16S rRNA, *gyrB, rpoD* and *gltA*. These loci are used for multilocus sequence typing (MLST) of *P. syringae* and other plant-associated bacteria (http://genome.ppws.vt.edu/cgi-bin/MLST/docs/MLST; (Sarkar & Guttman, 2012). Sequences used in the phylogenetic comparisons were downloaded from the NCBI database.

6. Phylogenetic analysis was performed using MEGA version 6.0 after multiple alignment of data by ClustalW (Thompson *et al.,* 1994). Distances (distance options according to Kimura's two-parameter model) and clustering with the neighbour-joining (Saitou & Nei, 1987) method were determined by using bootstrap values based on 1000 replications. Bootstrap majority-rule (> 50 %) consensus trees were obtained.

6.3 Results

6.3.1 Genome Characteristics

The complete genome of *P. poae* contains a circular chromosome that is 6,176,813 bp in size with a G+C content of 60.4 %. No plasmid was found. The first version of the annotation includes 3 rRNA genes, 46 tRNA genes, and 5,514 protein-coding genes (Table 6.1). The protein-coding genes have an average length of 981.86 bp and account for 88 % of the chromosome. Additionally, the *P. poae* genome has been compared with other plant based pseudomonad genome to underline similarities among them.

Table 6.1: Genome characteristics of 12 strains of plant-associated *Pseudomonas.*									
ID	**Strain**	**Size (Mb)**	**G+C**	**rRNAgenes**	**tRNA**	**Protein-coding genes**	**Plasmid**	**Property**	**References**
Ppoae24	***P. poae* PpR24**	6.17	60.4	3	46	5,514	-	Oral Insecticidal property, Biocontrol by secondary metabolites production	Livermore, 2016
PCL1751	***P. fluorescens* PCL1751**	6.1	60.4	19	70	5,534	-	Biocontrol by competition for nutrients and niches, plant growth-promotion, and increase of plant salt stress tolerance	Cho *et al.,* 2015
SBW25	***P. fluorescens* SBW25**	6.7	60.5	16	68	5,921	-	Plant growth-promotion	Silby *et al.,* 2009
A506	***P. fluorescens* A506**	5.96	60.0	19	69	5,267	1	Biocontrol	Loper *et al.,* 2012
UK4	***P. fluorescens* UK4**	6.03	60.1	19	68	5,178	-	Biofilm-forming and amyloid-producing	Dueholm *et al.,* 2014
Pf0-1	***P. fluorescens* Pf0-1**	6.43	60.5	19	73	5,722	-	Soil-dwelling commensal Plant growth-promotion	Silby *et al.,* 2009
UW4	**P. sp. UW4**	6.18	60.1	22	72	5,423	-	Plant growth-promotion	Duan *et al.,* 2013
F113	***P. fluorescens* F113**	6.8	60.8	16	66	5,862	-	Biocontrol by secondary metabolite production	Redondo-nieto *et al.*, 2013
Pf-5	***P. protegens* Pf-5**	7.07	63.3	16	71	6,108	-	Biocontrol by antibiotics production	Paulsen *et al.,* 2005
CHA0	***P. protegens* CHAO**	6.8	63.4	15	68	6,115	-	Biocontrol by antibiotics production	Jousset *et al.,* 2014
PA23	***P. chlororaphis* PA23**	7.12	62.6	16	68	6,179	-	Biocontrol by antibiotics production	Loewen *et al.,* 2014
DC3000	***P. syringae pv.tomato DC3000***	6.39	58.4	15	63	5,482	2	Phytopathogenic	Buell *et al.,* 2003

6.3.2 Phylogenetic analysis of *P. fluorescens* and Related Species

The phylogenetic analysis of the *P. fluorescens* group revealed the presence of two clades with at least five subgroups with strains previously classified as *P. fluorescens*, interspersed with strains classified in other species (Figure 6.1). In both phylogeny trees, PpR247 was observed to reside in subclade-1 and to be closely related to *Pseudomonas fluorescens* SS101, which was isolated from wheat roots in the Netherlands (Figure 6.1 & Appendix Figure 8). The sub clade-1 also includes previously sequenced *P. fluorescens* strains SBW25, A506, NZ052, PCL1571 and EGD-AQ6. The subclade-2 contains *P. fluorescens* strains WH6, BS2, NZ007 and BRIP34897, together related with *P. poae* RE strain. Total 10 P. *fluorescens* strains were clustered in Clade-2. The subclade-3 contains *P. protegens* Pf-5, CHAO and *P. fluorescens* strains Wayne1 clustered together. Subclades-4 and -5 contain the *P. fluorescens* F113, Pf0-1 and other strains respectively.

I also examined the genomes of *P. poae* and its closet relative, *Pseudomonas fluorescens* SS101 for the distribution of traits involved in plant microbe interaction and biocontrol (Table 6.2).

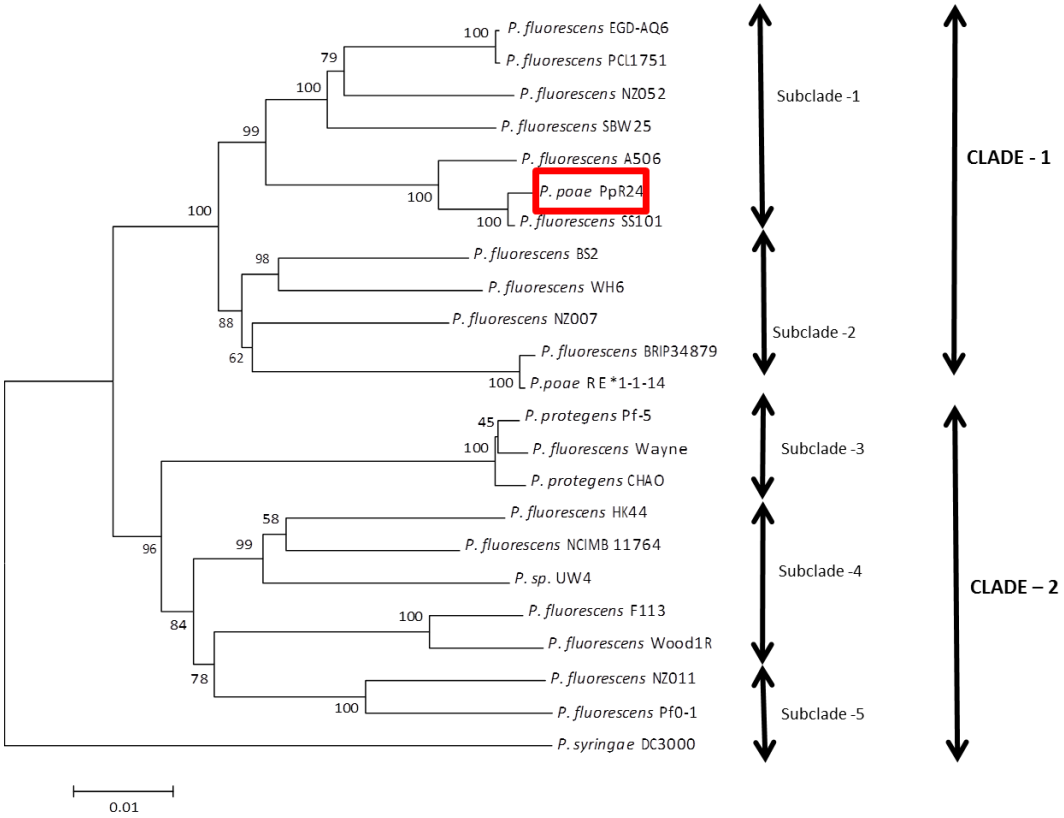


Figure 6.1: Neighbour-joining phylogenetic tree based on concatenated sequences of 16S rRNA, gyrB, rpoD and gltA genes.

The bootstrap values based on 1000 replications are depicted at the branch nodes. The scale bar depicts branch length of tree.

Table 6.2: Summary of selected biosynthetic/catabolic genes or gene clusters in the genome of both strains.		
Biosynthetic/ catabolic genes	***P. poae PpR24***	***P. fluorescens* SS101**
Antibiotics		
2,4-diacetylphloroglucinol (DAPG);	X	X
hydrogen cyanide (HCN)	X	X
Phenazine	✓	X
Rhizoxins	X	X
Pyoluteorin	X	X
2-hexyl-5-propyl-alkylresorcinol (HPR);	X	X
Cyclic lipopeptides		
Orfamide	X	X
Viscosin	X	X
Massetolide	X	✓
Unknown	X	X
Siderophores		
Pyoverdine	✓	✓
Enantio-pyochelin	X	X
Pseudomonine-like	X	X
Achromobactin	X	a
Hemophore	✓	✓
Orphan gene clusters		
PvfABCD	✓	✓
NRPS	✓	✓
Polyketide synthase (PKS	✓	✓
Bacteriocins		
Pyocin	✓	X
Colicin	✓	X
Microcin	X	✓
Plant bacterial communication		
Indole-3-acetic acid (IAA) biosynthesis	X	
Indole-3-acetic acid (IAA) catabolism	X	
Phenylacetic acid (PAA) Catabolism	X	X
Aminocyclopropane-1-carboxylic acid (ACC) deaminase	✓	✓
2,3-butanediol biosynthesis	✓	✓
Acetoin biosynthesis	✓	✓
Extracellular protease		
Chitinase	✓	✓
AprA	✓	✓
AprX	✓	X
Putative proteases	2	2
Secretion systems		
Type II Secretion systems		
Xcp	X	X

Hxc	X	X
Hxc-2	X	X
Novel	✓	✓
Type III Secretion systems	2	1
Type III effectors		
ExoU	✓	X
RpoB	X	X
RpoM	X	X
RpoAA-1	X	X
Inv/Mxi/Spa	X	X
Putative	3	15
Type IV Secretion systems	✓	X
Type VI Secretion systems	2	1
Toxins		
FitD	X	X
Tc complex	✓	✓
Chemotatic genes (CheZ gene clusters)	✓	✓
Key - "✓"presence of a gene or gene cluster within a genome, while " X " marks the absence of a cluster ; numbers represent the number of copies of a gene or cluster within a genome.		

6.3.3 Functional annotation of *P. poae* genome

Blast2GO is a popular annotation platform that uses results from homology searches to associate sequence with GO terms and other functional annotations. *P. poae* sequences were searched against the non-redundant database using BLASTx algorithms and the InterPro database and the results were imported into the Blast2GO program's graphical user interface, which assigned GO terms to 4,102 out of 5479 transcripts. The potential genes which are involved in virulence against aphids, plant growth promotion and other aspects of plant colonization are discussed below.

6.3.3.1 Insecticidal Toxins

The functional annotation of *P. poae* revealed several insecticidal toxins and virulence factors. In addition to this, a second screening for potential insecticidal genes in the *P. poae* genome was done via a database search engine designed by Prof. Primitivo Caballero's (Universidad Publica de Navarra) lab, which confirmed the presence of three different insecticidal toxins (Table 6.3).

Table 6.3: A list of Insecticidal toxin genes in the *P. poae* genome.	
Gene Locus	**Function**
Class -1 "Tc toxin complex"	
CDH05_22010	hypothetical protein
CDH05_22015	hypothetical protein
CDH05_22020	Insecticidal toxin complex protein TccC1
CDH05_22025	Insecticidal toxin complex protein TcaC1
CDH05_22030	Insecticidal toxin complex protein TcaB1
CDH05_22035	Insecticidal toxin complex protein TcaA1
Class -2 "Rhs family protein"	
CDH05_26805	Rhs family protein
CDH05_04585	Rhs-family protein
Class-3 "Metalloproteases"	
CDH05_13340	Secreted alkaline metalloproteinase (EC 3.4.24.-),(AprA)
CDH05_16530	Secreted alkaline metalloproteinase(EC 3.4.24.-), (AprX)

The *P. poae* genome contains genes that encode for major insecticidal toxin complex "Tc" proteins that have been found only in entomopathogenic enterobacteria such as *Photorhabdus luminescens, Serratia entomophila, Xenorhabdus nematophilus* or in *Yersinia spp* (Waterfield *et.al.,*2001; Bowen *et al.,* 1998). Three basic genetic elements encode insecticidal toxin complexes: *tcdA*-, *tcdB*- and *tccC* like genes. The *P. poae* genome encodes two TcA-like (TcaA1, TcaB1), one TcB-like (TcaC1) & one TcC-like (TccC2) insecticidal toxins. Tc protein gene clusters have been reported in the genomes of plant associated *Pseudomonas* strains (SS101, A506 & Q2-87) and their functions in the bacterial ecology is still not clear. To date, the role of the Tc complex in *Pseudomonas* spp. is limited to *P. taiwanensis* where TccC was heterologously expressed in *E. coli* and caused substantial mortality to *Drosophila* larva (Liu *et.al.,* 2010). The genome sequencing of *Ph. luminescens* around the tc loci revealed 15 hypothetical genes, which are potentially involved in virulence (Waterfield *et al.*, 2001)

Similarly, the presence of two hypothetical proteins (Hypr1 and Hypr2) in close proximity to the insecticidal toxin complex in the *P. poae* genome, suggests that they may have been acquired independently or been inherited together and will be transcribed at the same time. For this reason it may be hypothesized that the Hypr1 and Hypr2 play roles in insecticidal toxicity (Figure 6.2).

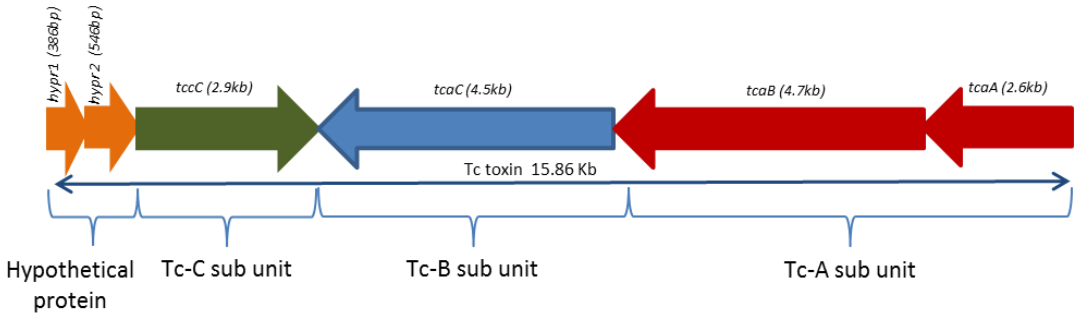


Figure 6.2: *P. poae* toxin complex (tc). Gene loci consist of three conserved toxin elements (TcA-like, TcB-like and TcC-like) are colour coded (red, blue and green, respectively). The open reading frames (*tcaA, tcaB,* and *tcaC*) transcribed in one direction and a short terminal open reading frame (*tccC*) transcribed in the opposite direction. Two hypothetical protein genes found closer to tc locus and transcribed in opposite direction.

Furthermore, InterPro results showed that the amino acid sequence of TcaA1 has a conserved domain called VRP1. The VRP1 domain corresponds to SpvA, the product of a plasmid-borne gene associated with virulence of *Salmonella spp* (Spink *et al.,* 1994). In TcaB1, the only detected motifs were two small coil regions. Three conserved SpvB, MidN and MidC (middle/ N & C-terminal) domains are found in TcaC1 which showed high similarity to domains present in the corresponding *Ph. luminescens* and *Yersinia pseudotuberculosis* insecticidal toxins (Yang & Waterfield, 2013). The TccC2 has a conserved RHS repeat, similar to that found inTccC1 from *Xenorhabdus*. The presence of a transmembrane helix domain in TccC2 indicates that the protein is likely active when residing within the cell membrane.

A second putative toxin (Rhs family protein) contains a YD-peptide repeats and has a core/core-extension architecture that is reminiscent of “rearrangement hotspot (Rhs)” were reported in *P. poae* genome. Two different gene loci of *P. poae* genome (CDH05_26805 & CDH05_04585) encode Rhs family protein (RhsA1 & RhsA2) which carry the same core extension toxin domains.

Rhs family proteins play an important role in bacterial interactions with eukaryotic host cells. Rhs like elements have been previously reported in toxin-complex C proteins (TccC) of *Ph. luminescens* and *S. entomophila* pathogenicity determinant C (SepC) of *S. entomophila* that are used to destroy the midgut of insect hosts (Hurst *et al.,* 2000; Waterfield *et al.*, 2001). Moreover, an rhs4T gene of *P. aeruginosa* encodes a toxic protein that activates the inflammasome-mediated death of host cells. RhsA and RhsB from *Dickeya dadantii* 3937 carry nuclease domains that degrade target plant cell DNA by exporting Rhs proteins using a T6S mechanism.

Similarly, in the *P. poae* genome two Rhs family proteins (RhsA1 & RhsA2) were suggested their role in the virulence against host cells.

Proteases another important member of extracellular, biologically active substances that assumed to contribute in the virulence of various bacterial species (Heermann & Fuchs, 2008). The *P. poae* genome encodes four serine proteases, two Zn-dependent metalloproteases (AprA and AprX) and other two proteases. The Zn-dependent metallopeptidase AprX, also called serralysin, and the AprA alkaline protease are actively degrading the diptercin antimicrobial peptide of drosophila during the early phase of bacterial infection (Liehl *et al.,* 2006). A third predicted toxin in *P. poae* (CDH05_13340 & CDH05_16530) was classified in metalloproteases category.

6.3.3.2 Metabolism, transport and regulation

The annotation of *P. poae* identified 135 genes which are putatively involved in carbohydrate, lipid, amino acid, nucleotide, vitamins and cofactor, and xenobiotic metabolism.

The *P. poae* genome encodes several central metabolic pathways found in the other members of the *Pseudomonas* species including the pentose phosphate pathway, the Entner-Doudoroff pathway and the tricarboxylic acid cycle. The *P. poae* genome harbors several genes that encode hydrolytic activities such as chitinases, lipases and proteases as well as a set of 19 uncharacterized hydrolases potentially involved in the degradation of macromolecules found in the environment.

The *P. poae* genome also carries genes for the catabolism of long-chain carbohydrates and several aromatic compounds. *P. poae* shares various genetic determinants with *P. putida* that are involved in the degradation of different types of aromatic compounds including phenylalanine, tyrosine,benzoate, quinate, 4-hydroxybenzoate as well as phenyl acetaldehyde and phenylalkanoate (Jiménez *et al.,* 2002).

P. poae also has an extended collection of metabolite efflux systems, with 12 Drug/Metabolite Transporter (Dmt) family metabolite efflux pumps and 19 Resistance to Homoserine/ Threonine (RhtB) family amino acid efflux pumps, which are potentially involved in protection against toxic effects of metabolites or metabolic analogues.

Table 6.4: A list of Secretion Systems in the *P. poae* genome.	
Gene Locus	**Function description**
Type I Secretion System (two copy of T1SS at different gene locus)	
CDH05_24305	Type I secretion outer membrane protein, TolC precursor
CDH05_13630	Type I secretion outer membrane protein, TolC precursor
Type II Secretion System (two copy of T2SS at different gene locus)	
CDH05_22675	Type II/IV secretion system protein TadC, associated with Flp pilus assembly
CDH05_22685	Type II/IV secretion system ATP hydrolase TadA/VirB11/CpaF, TadA subfamily
CDH05_22695	Type II/IV secretion system secretin RcpA/CpaC, associated with Flp pilus assembly
CDH05_03410	Type II secretory pathway, ATPase PulE/Tfp pilus assembly pathway, ATPase PilB
Type III Secretion System (whole T3SS gene complex & its effector proteins)	
CDH05_20550	HrpL
CDH05_20555	Type III secretion protein HrpJ
CDH05_20560	Type III secretion inner membrane channel protein (LcrD, HrcV, EscV, SsaV)
CDH05_20565	type III secretion protein HrpQ
CDH05_20570	Flagellum-specific ATP synthase FliI
CDH05_20575	hypothetical protein
CDH05_20580	Type III secretion protein HrpP
CDH05_20585	Type III secretion inner membrane protein (YscQ, homologous to flagellar export components)
CDH05_20590	Type III secretion inner membrane protein (YscR, SpaR, HrcR, EscR, homologous to flagellar export components)
CDH05_20595	Type III secretion inner membrane protein (YscS, homologous to flagellar export components)
CDH05_20600	Type III secretion inner membrane protein (YscT, HrcT, SpaR, EscT, EpaR1,homologous to flagellar export components)
CDH05_20605	Type III secretion inner membrane protein (YscU, SpaS, EscU, HrcU, SsaU, homologous to flagellar export components)
CDH05_20610	negative regulator of hrp expression HrpV
CDH05_20615	type III secretion protein HrpT
CDH05_20620	Type III secretion outermembrane pore forming protein (YscC, MxiD, HrcC, InvG)
CDH05_20625	hypothetical protein
CDH05_20635	Type III secretion cytoplasmic protein (YscL)
CDH05_20630	hypothetical protein
CDH05_20645	Type III secretion bridge between inner and outermembrane lipoprotein (YscJ, HrcJ, EscJ, PscJ)
CDH05_20650	hypothetical protein
CDH05_20655	hypothetical protein
CDH05_20660	type III transcriptional regulator HrpR
CDH05_17280	Type III effector HopPmaJ
CDH05_02660	TypeIII secretion system effector protein ExoU
CDH05_21135	Putative T3SS secretion effector protein
CDH05_18485	Putative T3SS secretion effector protein
CDH05_18475	Putative T3SS secretion effector protein
CDH05_15660	Putative T3SS secretion effector protein
CDH05_15655	Putative T3SS secretion effector protein
Type IV Secretion System (two copy of T4SS at different gene locus)	
CDH05_12435	Type IV secretory pathway, VirJ component
Type VI Secretion System (two copy of T6SS at different gene locus)	
CDH05_04595	**VgrG protein**

CDH05_04600	ClpB protein
CDH05_04605	Uncharacterized protein ImpH/VasB
CDH05_04610	Protein ImpG/VasA
CDH05_04615	Uncharacterized protein ImpF
CDH05_04620	Uncharacterized protein ImpD
CDH05_04625	Uncharacterized protein ImpC
CDH05_04630	Uncharacterized protein ImpB
CDH05_04635	Uncharacterized protein ImpA
CDH05_04640	Uncharacterized protein ImpI/VasC
CDH05_04645	Type VI secretion lipoprotein/VasD
CDH05_04650	Uncharacterized protein ImpJ/VasE
CDH05_04655	Outer membrane protein ImpK/VasF, OmpA/MotB domain
CDH05_04660	IcmF-related protein
CDH05_04665	Protein phosphatase ImpM
CDH05_04670	Phosphoprotein phosphatase PppA
CDH05_04675	Serine/threonine protein kinase (EC 2.7.11.1) PpkA
CDH05_19235	**Secreted protein Hcp**
CDH05_19230	Uncharacterized protein ImpA
CDH05_19225	Uncharacterized protein ImpB
CDH05_19220	Uncharacterized protein ImpC
CDH05_19215	Uncharacterized protein similar to VCA0109
CDH05_19210	Protein ImpG/VasA
CDH05_19205	Uncharacterized protein ImpH/VasB
CDH05_19200	Sigma-54 dependent transcriptional regulator
CDH05_19195	hypothetical protein
CDH05_19190	hypothetical protein
CDH05_19185	Type VI secretion lipoprotein/VasD
CDH05_19180	Uncharacterized protein ImpJ/VasE
CDH05_19175	Outer membrane protein ImpK/VasF, OmpA/MotB domain
CDH05_19170	IcmF-related protein

Bacteria depend on several secretion systems to communicate with the extracellular environment for survival. The genome of *P. poae* contains a wide variety of secretion systems, which include two T1SSs, two T2SSs, one T3SS, two T4SSs, and two T6SS (Table 6.4).

Two genes of T1SS encoding outer membrane protein "TolC" were found in *P. poae* genome. These TolC proteins may be involved in the export of virulence proteins and toxins without any periplasmic intermediate (Koronakis *et al.,* 1997). In the *P. poae* genome, a new subtype of T2SS Tad (tight adherence) that encode the machinery for biofilm formation colonization and pathogenesis were found (Tomich *et al.,* 2007).

The annotation of *P. poae* T3SS gene clusters showed the maximum similarity to *Pseudomonas fluorescens* strain KENGFT3 T3SS gene cluster, which suggested it is a plant *hrp-hrc* type T3SS gene system in *P. poae* genome.

In the *P. poae* genome, genes encoding outer and inner membrane proteins of a type III secretion injectisome assembly –*hrcC, hrcJ, hrpE, hrpT* (CDH05_20615, CDH05_20620,

CDH05_20635, & CDH05_20645) were found. Five essential integral membrane genes of injectisome – *hrcQ, R*, S, T, U (CDH05_20585, CDH05_20590, and CDH05_20595 & CDH05_205600) - were found which form the export channel across the inner membrane. Besides, other genes which are involved in exporting the needle tip and regulation (*hrpP*, ATP synthase (*hrcN*)) of effector proteins transport from the bacterial cell into host cells were observed. Additionally, two positive regulators HrpR and HrpL, related to activators of hypersensitive response and pathogenicity (Hrp) system of *P. syringae,* were identified in the *P. poae* genome (Thwaites *et al.,* 2004). A negative regulator of the Hrp system termed as HrpV was also observed in the genome (Figure 6.3 & Table 6.5) (Preston *et al.,* 1998).

Table 6.5: Summary of homologous core components of the T3SS in *P. poae* genome.		
Core components of the T3SS	**Genes found in *P. poae* Genome**	**Predicted Function**
Core channel components	HrcR,S,T,U HrcN	Membrane channel Energy source for protein transport or folding
Basal body	HrpQ HrcJ	Basal body complex MS ring structure of basal body
Outer ring components	HrcC HrpT	Outer membrane protein Chaperons for HrcC
Accessory protein or regulators	HrpP HrpE HrpJ	Needle tip regulator Sorting platform Export regulator
(Nomenclature Of T3SS gene cluster - *hrp /hrc* (*Pseudomonas* spps.)		

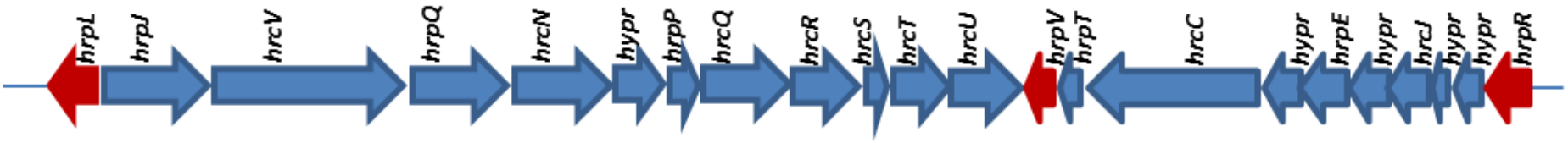


Figure 6.3: *P. poae* type III secretion system. Gene loci consist of 19 open reading frames of structural components of T3SS in the *P. poae* genome. Other three genes (*hrpL, hrpR* & *hrpV*) with major regulatory functions are shaded in red boxes. The black arrows indicated the transcription direction in the gene cluster. {Nomenclature of T3SS gene cluster - *hrp /hrc* (*Pseudomonas spps.)* Hypr= hypothetical protein}.

Annotation of the *P. poae* genome identified the following putative effectors: homolog of *P. syringae* effector HopAS1 (CDH05_17280) and other ExoU (CDH05_02660) effector belong to *P. aeruginosa*. Other putative T3SS effectors were also reported in the *P. poae* genome. Furthermore, the identification of the *exoU* effector gene within *P. poae* potentially indicates that the type III secretion system could be associated with the bacterium's pathogenic effect on aphids. It has been shown that ExoU is one of the main *P. aeruginosa* effectors to be secreted, and it is associated with bacterial dissemination and sepsis in animal models and human infections (Sato *et al.,* 2003). Cell death is caused by ExoU's phospholipase activity with broad substrate activity. This effector plays an integral role in *P. aeruginosa's* pathogenicity in *G. mellonella* (Miyata *et al.,*2003). It is reasonable to therefore consider that this effector could therefore be involved in *P. poae's* pathogenicity in aphids.

The type VI secretion system (T6SS) is another specialized system which allows the bacteria to interact with their environment and other cells via a needle-like apparatus. It has been suggested that the type VI system may be involved in the commensal or mutualistic relationships between bacteria and eukaryotic host, and mediate the function of cooperation or competition in inter-bacterial interactions (Jani & Cotter, 2010; Decoin *et al.*, 2014).

Two different T6SS gene clusters defined as "hemolysin co-regulated protein (Hcp)" (CDH05_19235) and "VgrG, or Valine-Glycine Repeat Protein G" (CDH05_04595) have been identified in the *P. poae* genome. The occurrence of such secretion systems could assist *P. poae* to survive and compete in planta, and within the aphids gut.

The *P. poae* genome encodes a complex array of regulatory systems including 18 predicted sigma factors, more than 276 genes encoding predicted transcriptional regulators and a variety of two-component signal transduction systems consisting of histidine kinase domains and response regulator domain.

6.3.3.3 Other virulence and niche adaptation traits

Many strains of *P. fluorescens* are well known to produce diverse secondary metabolites with antifungal and antibacterial properties (Haas & Keel, 2003; Raaijmakers *et al.,* 2010; Jousset *et al.*, 2011). These metabolites are essential for competition and survival in the rhizosphere and led foundation of biocontrol activities. The *P. poae* genome carries genes that are similar to those involved in the antibiotic synthesis such as phenazine and mitomycin (Table 6.6). The phenazines could cause virulence to insects, by modifying cellular redox states, which act as cell signals that regulate patterns of gene expression, contribute to biofilm formation and architecture, and enhance bacterial survival (Wang *et al.*, 2011). In the *P. poae* genome, genes required for pyoverdine biosynthesis and uptake are present. With the identification

of these proteins it would seem that *P. poae* is capable of iron acquisition, which is likely to be important within an animal host (Ochsner *et al.,* 2002).

To defend against reactive oxygen species, many antioxidant enzymes (superoxide dismutase [SOD], catalase, and peroxidase), iron uptake and transport, DNA binding proteins, DNA repair enzymes and free-radical-scavenging agents have been reported in most Pseudomonads (Ma *et al.,* 1999; Ochsner *et al.,* 2000; Shin *et al.,* 2007; Kang *et al.,* 2017).

Genes encoding putative proteins involved in scavenging reactive oxygen species including two superoxide dismutases, six catalases, 11 peroxidases (one cytochrome C peroxidases, three glutathione peroxidases, four dyp-type peroxidases, two thiol peroxidases and four alkyl hydroperoxidases) are also found in the *P. poae* genome (Table 6.7). In the *P. poae* genome, major regulators of oxygen reactive species such as MarR-family redox sensors: PqrR and OhrR and LysR regulator "OxyR" which were similar to *P. aeruginosa* regulators were reported (Cornelis *et al.,*2011).

To protect DNA from reactive oxygen species, DNA binding and repair genes are also present in the *P. poae* genome. The presence of several genes conferring tolerance to oxidative stress in the genome of *P. poae* supports the proposed importance of oxidative stress tolerance to fitness inside diverse host environments. In addition, a number of other putative toxins and virulence factors are present in *P. poae* genome including three predicted hemolysin/haemagglutinins, two adhesin or agglutination proteins, two RTX toxins and four Rhs-family proteins (Table 6.8).

Table 6.6: A list of secondary metabolite genes, including those for pyoverdine synthesis, in the *P. poae* genome

Gene locus	Function
Pyoverdine sysnthesis and receptors	
CDH05_05880	Outer membrane ferripyoverdine receptor
CDH05_05885	Outer membrane porin, coexpressed with pyoverdine biosynthesis regulon
CDH05_21475	Hypothetical protein, coexpressed with pyoverdine biosynthesis regulon
CDH05_21935	Pyoverdine-specific efflux *macA*-like protein
CDH05_21940	Pyoverdine efflux carrier and ATP binding protein
CDH05_21945	Outer membrane pyoverdine eflux protein
CDH05_05850	Pyoverdine biosynthesis related protein PvdP
CDH05_05855	PvdO, pyoverdine responsive serine/threonine kinase
CDH05_05870	Pyoverdine synthetase PvdF, N5-hydroxyornithine formyltransferase
CDH05_05875	PvdE, pyoverdine ABC export system, fused ATPase and permease components
CDH05_05880	Outer membrane ferripyoverdine receptor FpvA, TonB-dependent
CDH05_05885	Pyoverdine sidechain non-ribosomal peptide synthetase PvdD
CDH05_21470	Outer membrane porin, coexpressed with pyoverdine biosynthesis regulon
CDH05_00385	Pyoverdine chromophore precursor synthetase PvdL
Antibiotic synthesis	
CDH05_27105	Protein involved in biosynthesis of mitomycin antibiotics/polyketide fumonisin
CDH05_26820	Phenazine biosynthesis protein PhzF like

CDH05_23055	Aminodeoxychorismate lyase (EC 4.1.3.38)
CDH05_20540	Phenazine biosynthesis protein PhzF
CDH05_04555	Chorismate--pyruvate lyase (EC 4.1.3.40)
CDH05_09165	Periplasmic chorismate mutase I precursor (EC 5.4.99.5)
CDH05_00605	Chorismate synthase (EC 4.2.3.5)
CDH05_10100	Chorismate mutase I (EC 5.4.99.5) / Prephenate dehydratase (EC 4.2.1.51)

Table 6.7: A list of oxidative stress genes in the *P. poae* genome.

Gene locus	Function
CDH05_15375	Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)
CDH05_15370	Alkyl hydroperoxide reductase protein F (EC 1.6.4.-)
CDH05_25870	Alkyl hydroperoxide reductase subunit C-like protein
CDH05_03370	Alkyl hydroperoxide reductase subunit C-like protein
CDH05_25140	4-carboxymuconolactone decarboxylase domain/alkylhydroperoxidase AhpD family core domain protein
CDH05_21000	Superoxide dismutase [Fe] (EC 1.15.1.1)
CDH05_10530	Manganese superoxide dismutase (EC 1.15.1.1)
CDH05_11270	Catalase (EC 1.11.1.6)
CDH05_12660	Catalase (EC 1.11.1.6)
CDH05_20210	Catalase (EC 1.11.1.6)
CDH05_07610	Catalase (EC 1.11.1.6)
CDH05_20375	Mn-containing catalase
CDH05_03565	Catalase (EC 1.11.1.6)
CDH05_19635	Thiol peroxidase, Tpx-type (EC 1.11.1.15)
CDH05_22400	Thiol peroxidase, Bcp-type (EC 1.11.1.15)
CDH05_17145	Cytochrome c551 peroxidase (EC 1.11.1.5)
CDH05_17315	Glutathione peroxidase (EC 1.11.1.9)
CDH05_17750	Glutathione peroxidase (EC 1.11.1.9)
CDH05_04795	Glutathione peroxidase (EC 1.11.1.9)
CDH05_01050	Non-haem chloroperoxidase (EC 1.11.1.10)
CDH05_24265	Ferrous iron transport peroxidase EfeB
CDH05_11515	Predicted dye-decolorizing peroxidase (DyP), YfeX-like subgroup
CDH05_24570	Predicted dye-decolorizing peroxidase (DyP), encapsulated subgroup

Table 6.8: A list of other potential virulence genes in the *P. poae* genome.

Gene locus	Function
CDH05_18130	Extracellular serine protease precursor (EC 3.4.21.-)
CDH05_16510	Secreted alkaline metalloproteinase (EC 3.4.24.-), PrtA/B/C/G homolog
CDH05_16555	Serine protease homologue
CDH05_16560	Serine protease homologue
CDH05_23010	Periplasmic serine proteases (ClpP class)
CDH05_18885	Secreted alkaline metalloproteinase (EC 3.4.24.-), PrtA/B/C/G homolog
CDH05_25340	Large exoproteins involved in haem utilization or adhesion
CDH05_26805	Rhs family protein
CDH05_24425	Rhs-family protein
CDH05_24430	Rhs family protein, putative
CDH05_21710	21 kDa hemolysin precursor
CDH05_02730	COG1272: Predicted membrane protein hemolysin III homolog
CDH05_01905	Phospholipase/lecithinase/hemolysin
CDH05_07860	Agglutination protein
CDH05_18645	Urease alpha subunit (EC 3.5.1.5)
CDH05_18650	Urease beta subunit (EC 3.5.1.5)
CDH05_18665	Urease gamma subunit (EC 3.5.1.5)

CDH05_18670	Urease accessory protein UreD
CDH05_18675	Urea ABC transporter, ATPase protein UrtE
CDH05_18680	Urea ABC transporter, ATPase protein UrtD
CDH05_18685	Urea ABC transporter, permease protein UrtC
CDH05_18690	Urea ABC transporter, permease protein UrtB
CDH05_18695	Urea ABC transporter, substrate binding protein UrtA
CDH05_13990	Urease accessory protein UreG
CDH05_13995	Urease accessory protein UreF
CDH05_14000	Urease accessory protein UreE
CDH05_18680	Urea ABC transporter, ATPase protein UrtD
CDH05_01085	Chitinase (EC 3.2.1.14)
CDH05_01080	Chitin binding protein

6.3.3.4 Chemotaxis, Motility, Adhesion, and Other Aspects of Root Colonization

As expected, for a rhizobacteria that exhibits strong competitive colonization ability of plant roots, I identified clusters genes related to chemotaxis, motility, and adhesion (Kamilova *et al.,* 2005).

For chemotaxis, only the Che (*cheA*, *cheB*, *cheR*, *cheW*, *cheY*, and *cheZ*) systems for signal transduction were present and the Wsp system (that influences expression of extracellular polysaccharide *wss* genes), were absent (Table 6.9). Notably, 25 copies of the methyl-accepting chemotaxis protein genes (*mcp*) were found, suggesting that this bacterium has a wide range of trans-membrane sensor proteins for different signals (Table 6.8). For motility, the genes involved in the regulation (*fleQ, fleR,* and fleS), biosynthesis (*flhA, flhB, flhF, flhG, fliP, fliQ*, and *fliR*), structure (*flgA, flgB, flgC, flgD, flgE, flgF, flgG, flgH*, *flgI, flgJ, flgK, flgL, fliC, fliD, fliE, fliF, fliG, fliH, fliI, fliJ, fliK, fliL, fliM, fliN, fliO, fliS*, and *fliT*), and motor (*mot*A and *mot*B) components of flagella were found (Table 6.10). Other polysaccharide biosynthetic genes such as alginate and lipopolysaccharide were reported.

Table 6.9: A list of chemotaxis and motility genes in the *P. poae* genome.

Gene locus	Function
CDH05_28270	Methyl-accepting chemotaxis protein
CDH05_18580	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_14300	Methyl-accepting chemotaxis protein
CDH05_23645	Methyl-accepting chemotaxis transducer
CDH05_18580	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_24345	Methyl-accepting chemotaxis protein
CDH05_16800	Methyl-accepting chemotaxis protein
CDH05_06315	Methyl-accepting chemotaxis protein
CDH05_06705	Methyl-accepting chemotaxis protein
CDH05_15730	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_23395	Methyl-accepting chemotaxis protein
CDH05_17970	Methyl-accepting chemotaxis protein
CDH05_19100	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)

CDH05_20005	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_26370	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_13800	Methyl-accepting chemotaxis protein
CDH05_17385	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_24235	Methyl-accepting chemotaxis protein
CDH05_10920	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_14880	Methyl-accepting chemotaxis protein
CDH05_02800	Chemotaxis signal transduction protein
CDH05_08995	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_08990	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
CDH05_01210	Methyl-accepting chemotaxis protein
CDH05_15705	Chemotaxis response regulator protein-glutamate methylesterase 1 (EC 3.1.1.61)
CDH05_24490	Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)
CDH05_15715	Chemotaxis signal transduction protein
CDH05_15515	Chemotactic transduction protein chpE
CDH05_28345	Chemotaxis protein CheV (EC 2.7.3.-)
CDH05_16180	Chemotactic transducer
CDH05_27165	Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)
CDH05_27160	Chemotaxis protein CheV (EC 2.7.3.-)
CDH05_00300	Chemotaxis protein CheV (EC 2.7.3.-)
CDH05_00270	Positive regulator of CheA protein activity (CheW)
CDH05_00265	CheW domain protein
CDH05_00245	Chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61)
CDH05_00240	Signal transduction histidine kinase CheA (EC 2.7.3.-)
CDH05_00235	Chemotaxis response - phosphatase CheZ
CDH05_00230	Chemotaxis regulator - transmits chemoreceptor signals to flagelllar motor components CheY

Table 6.10: A list of flagellar and polysaccharide genes in the *P. poae* genome.

Gene locus	**Function**
CDH05_16075	Sodium-type flagellar protein motY precursor
CDH05_13715	Flagellar motor rotation protein MotB
CDH05_13710	Flagellar motor rotation protein MotA
CDH05_27185	Flagellar hook protein FlgE
CDH05_27180	Flagellar basal-body rod modification protein FlgD
CDH05_27175	Flagellar basal-body rod protein FlgC
CDH05_27170	Flagellar basal-body rod protein FlgB
CDH05_27155	Flagellar basal-body P-ring formation protein FlgA
CDH05_27150	Negative regulator of flagellin synthesis FlgM
CDH05_27145	Flagellar biosynthesis protein FlgN
CDH05_00145	Flagellar biosynthesis protein FliL
CDH05_00255	Flagellar motor rotation protein MotB
CDH05_00250	Flagellar motor rotation protein MotA
CDH05_00225	RNA polymerase sigma factor for flagellar operon
CDH05_00220	Flagellar synthesis regulator FleN
CDH05_00215	Flagellar biosynthesis protein FlhF
CDH05_00210	Flagellar biosynthesis protein FlhA
CDH05_00180	Flagellar biosynthesis protein FlhB
CDH05_00175	Flagellar biosynthesis protein FliR
CDH05_00170	Flagellar biosynthesis protein FliQ
CDH05_00165	Flagellar biosynthesis protein FliP

CDH05_00160	Flagellar biosynthesis protein FliQ
CDH05_00155	Flagellar motor switch protein FliN
CDH05_00150	Flagellar motor switch protein FliM
CDH05_00145	Flagellar biosynthesis protein FliL
CDH05_00140	Flagellar hook-length control protein FliK
CDH05_00115	Flagellar protein FliJ
CDH05_00105	Flagellar assembly protein FliH
CDH05_00095	Flagellar M-ring protein FliF
CDH05_00090	Flagellar hook-basal body complex protein FliE
CDH05_00075	Flagellar regulatory protein FleQ
CDH05_00080	Flagellar sensor histidine kinase FleS
CDH05_00085	Flagellar regulatory protein FleQ
CDH05_00065	Flagellar biosynthesis protein FliS
CDH05_00060	Flagellar hook-associated protein FliD
CDH05_00040	Flagellar hook-associated protein FlgL
CDH05_00035	Flagellar hook-associated protein FlgK
CDH05_00030	Flagellar protein FlgJ [peptidoglycan hydrolase] (EC 3.2.1.-)
CDH05_00025	Flagellar P-ring protein FlgI
CDH05_00020	Flagellar L-ring protein FlgH
CDH05_00015	Flagellar basal-body rod protein FlgG
CDH05_00010	Flagellar basal-body rod protein FlgF
Polysaccharide genes	
CDH05_18195	Probable poly (beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
CDH05_18190	Alginate lyase precursor (EC 4.2.2.3)
CDH05_18185	Alginate biosynthesis protein AlgX
CDH05_18180	Poly (beta-D-mannuronate) C5 epimerase precursor (EC 5.1.3.-)
CDH05_18175	outer membrane protein AlgE
CDH05_18170	Alginate biosynthesis protein AlgK precursor
CDH05_18165	Alginate biosynthesis protein Alg44
CDH05_18155	Alginate biosynthesis protein Alg8
CDH05_18150	GDP-mannose 6-dehydrogenase (EC 1.1.1.132)
CDH05_13375	Exopolysaccharide production protein ExoZ
CDH05_10205	lipopolysaccharide biosynthesis protein
CDH05_13515	Lipopolysaccharide heptosyltransferase I (EC 2.4.1.-)
CDH05_18355	Glycosyl transferase, group 1 family protein
CDH05_10495	Lipopolysaccharide ABC transporter, ATP-binding protein LptB
CDH05_10490	LptA, protein essential for LPS transport across the periplasm
CDH05_10485	Uncharacterized protein YrbK clustered with lipopolysaccharide transporters
CDH05_10480	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase (EC 3.1.3.45)
CDH05_27045	Hexuronate transporter
CDH05_15020	Phosphomannomutase (EC 5.4.2.8) / Phosphoglucomutase (EC 5.4.2.2)
CDH05_14735	alginate regulatory protein AlgP
CDH05_18190	Alginate lyase precursor (EC 4.2.2.3)

6.3.4 Cellular infection and Secreted toxin

In the literature, three different routes of pathogenicity were stated for insects, involving invasion, gut occlusion and/or secretion of toxins.

The first stage of microbial infection is colonization where pathogens usually colonize in host tissue (such as digestive tract) by adherence factors and some ability to counter host defences at the surface.

During invasion, *D. dadantii* is able to replicate in aphid guts and produce extracellular toxins and enzymes (lecithinases, phospholipases and proteases) to act on the midgut cells, disrupt the epithelial barrier and invade the haemocoel, resulting in septicemia (Costechareyre *et al.,* 2012).

The gut occlusion is another mode of infection where *Pantoea stewartii* is able to effectively replicate inside gut, using essential nutrients, and potentially creating cell aggregates and blocking the aphids gut. Due to blockage of the gut, an excess of sucrose means that it is not secreted in the honey dew and aphids automatically stop eating and eventually starve to death (Stavrinides *et al.,* 2009).

Toxin mediated death occurs when the bacteria secretes a protein or a toxin that causes death, which is reported in *B. thuringiensis* (Cry toxin) *& Ph. luminescens* (Tc toxin) (Wu *et al.,*. 1997; Yang & Waterfield, 2013).

Experiments were carried out to identify if the pathogenic effect observed is likely due to being caused by a cellular infection or by a secreted product i.e. a toxin or protein. The basis of this was to test bacterial culture filtrate to determine whether a secreted product might be present and able to kill the aphids. Bacteria were first grown in both Mittler diet and LB media for 18, 24, 36, 48, 60 & 72 h at 20 $^{\circ}$C growth conditions. The cells were separated from the Mittler diet by filtration and the filtrate of the diet used to test for aphid mortality. Similarly, washed bacteria cells adjusted to 10^7 CFU mL^{-1} of same time points were used to perform the aphid mortality assay with sterile water as blank control. The aphid mortality readings were recorded for 72 h. Additionally, to evaluate any secreted protein (or toxin) by *P. poae* during infection, the total amount of protein was calculated at all time point filtrates of both media. I found a minimal amount of protein in control (filtrate without bacteria), therefore, this control protein amount was excluded from *P. poae* treated filtrates. The results showed no death from any of the tested filtrates (both LB and Mittler diet) during 72 h of observation. All washed bacteria cells (10^7 CFU mL^{-1}) of both LB and Mittler diet from each time sets were equally pathogenic to aphid over period of incubation (Table 6.11). No

death was observed with the control. The amount of protein was higher in Mittler diet filtrate than LB media filtrate at all tested time points. However, I reported protein concentration continuously increasing in both filtrates during course of observation (Figure 6.4). Moreover, linking the above findings with the growth curve of bacteria inside aphids, these data suggest that the ingestion of bacterial cells caused death, as it was able to replicate inside aphid gut (Figure 6.5).

Table 6.11: Assessment of aphid mortality by bacterial cells.

Description	Aphid mortality (Mean) recorded at different h		
	24 h	48 h	72 h
Blank control (All time points)	0	0	0
Filtrate from Mittler diet (18,24,36,48,60&72 h)	0	0	0
Filtrate from LB media (18,24,36,48,60&72 h)	0	0	0
Washed cells from Mittler diet grown at 18 h	0	90	100
Washed cells from Mittler diet grown at 24 h	0	95	100
Washed cells from Mittler diet grown at 36 h	0	90	100
Washed cells from Mittler diet grown at 48 h	0	95	100
Washed cells from Mittler diet grown at 60 h	0	100	100
Washed cells from Mittler diet grown at 72 h	0	100	100
Washed cells from LB grown at 18 h	0	95	100
Washed cells from LB grown at 24 h	0	95	100
Washed cells from LB grown at 36 h	0	95	100
Washed cells from LB grown at 48 h	0	95	100
Washed cells from LB grown at 60 h	0	100	100
Washed cells from LB grown at 72 h	0	100	100
Mortality assay showing the percentage of dead aphids (N=10) at different h after ingestion of Mittler diet inoculated with washed bacterial cells (10^7 CFU mL^{-1}), filtrate and blank control			

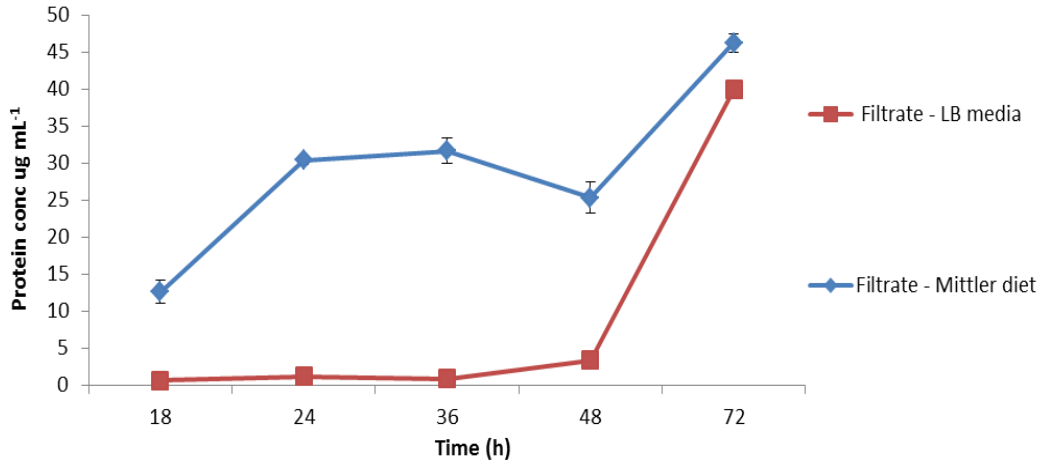


Figure 6.4: Protein estimation on the filtrate of both media by Bradford assay. The data showed amount of protein (ug mL^{-1}) detected in filtrate of Mittler diet and LB media at 18, 24, 36, 48, 72 h. Error bars represent the standard error of the mean of three biological replicates.

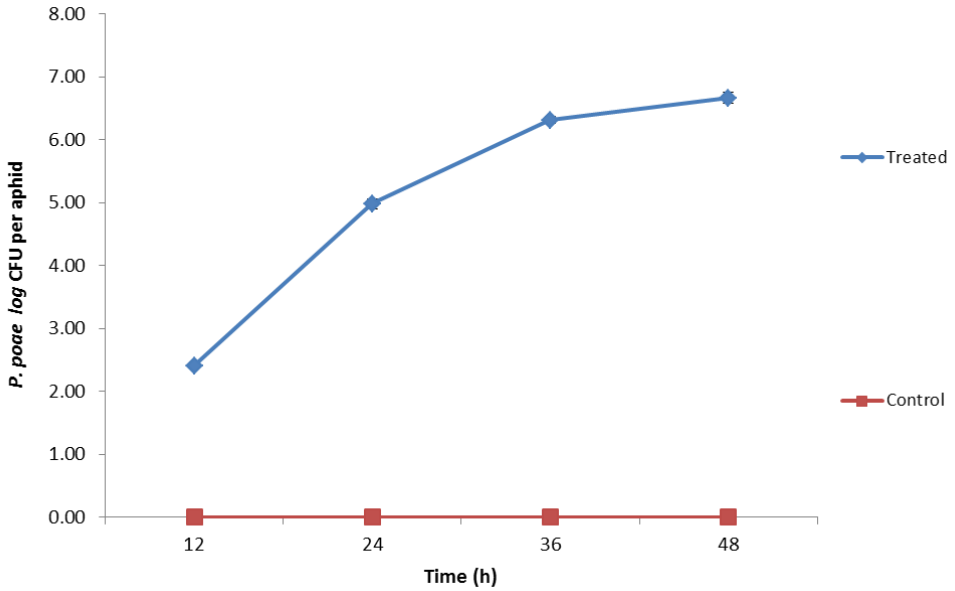


Figure 6.5: *P. poae* population growth inside aphid clone 4106A. *P. poae* populations within infected 4106A aphids were continually elevated to 6.66 $\log_{10}$ CFU aphid^{-1} over the period of inoculation and no colonies were recovered from control aphids for the entire duration of the experiment. **Control**: Ten aphids were fed in sterile diet with three replicates. **Treated**: Ten aphids, infected with 10^7 CFU mL^{-1}. *P. poae* in sterile diet with three replicates. Error bars represent standard error of the mean.

6.3.5 Gene Mutagenesis and complementation

To unravel *P. poae* virulence traits against aphids, a gene mutagenesis approach was employed. Firstly, a *P. poae* transposon mutant library was generated and screened to identify mutants altered in their ability to kill aphids. After screening 768 of *P. poae* mutants in an aphid mortality assay, no mutants were identified that showed altered timing of killing or loss of toxicity.

However, the *P. poae* genome analysis identified potential insecticidal toxins that might contribute in aphid pathogenesis. Moreover, the RNA-Seq analysis revealed higher expression of *aprX* and *hypr* genes and virulence traits suggested the role of toxins in pathogenicity.

I therefore employed gene deletion mutagenesis by the allelic exchange method (Merlin, *et al.*, 2002) on selected toxin genes (*tcAB, tcaA, rhsA2, aprX, hypr*). The absence of selected toxin gene after deletion mutagenesis was confirmed by PCR using primers outside the gene region, described in table 2.5 (Figure 6.6). To examine any effect on aphid mortality rate, the assays were performed with mutant and wild type cells, with an infection dose of 10^7 CFU mL^{-1}. The mean values of all different aphid mortality phenotypes were tested by two-way ANOVA followed by comparison of means by Tukey-Kramer HSD test using GenStat software.

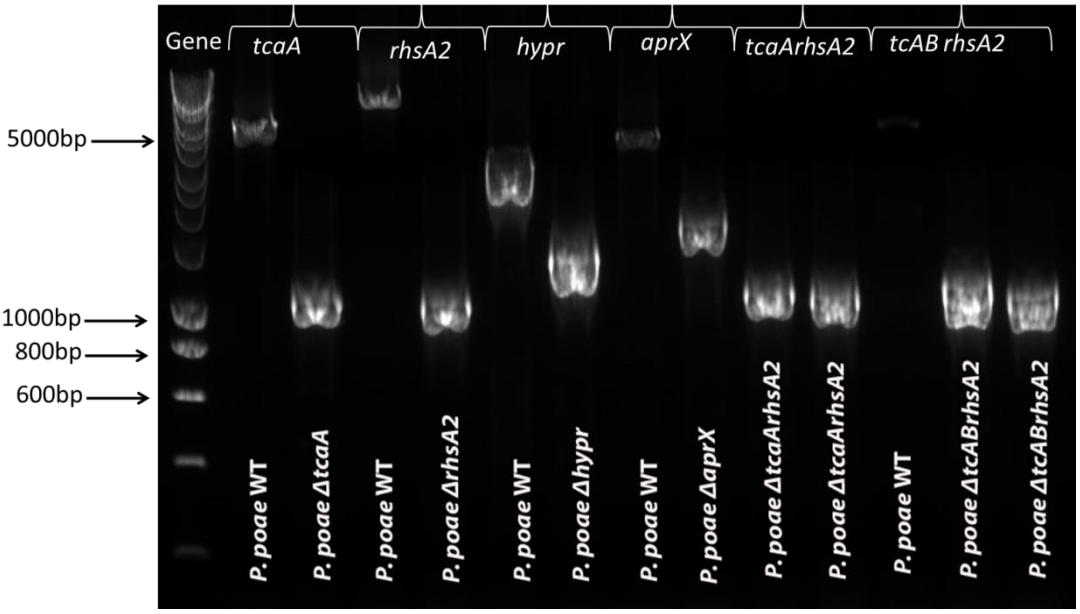


Figure 6.6: PCR amplifications of wild-type and deleted regions for five different toxin genes.
Amplification in all cases used the appropriate N_{ext}-C_{ext} primer pair. DNA size standards (lanes L) are on the first lane of gel, with length in base pairs, indicated on the left. The DNA band sizes are described as follows - *tcaA* gene WT = 3.9kb & *ΔtcaA* = 1500bp, *rhsA2 gene WT* = 5204 bp & *ΔrhsA2* = 1798bp*, aprX gene* WT = 3.1kb & *ΔaprX = 1647bp, hypr gene WT* = 2.8kb & *Δhypr = 1470b.* In case of dou*ble* mutants*, ΔtcaArhsA2* both *tcaA &rhsA2 genes* deleted*; ΔtcaA = 1500bp & ΔrhsA2 = 1798bp, ΔtcABrhsA2* both *tc-AB subunit genes & rhsA2* genes deleted therefore for *tcAB gene WT = 12kb & ΔtcAB = 1500bp & ΔrhsA2 = 1798bp.*

It was observed that deletion of *tcaA, rhsA2 and hypr* to create *ΔtcaA, ΔrhsA2, Δhypr* caused significant reductions in aphid mortality by an average of 50 % rate; all these mutants took 68-72 h to kill aphids, which were 48 h' longer than the time taken by wild type (Figure 6.8). However, the most significant decrease in aphid mortality was seen after deletion of the metalloprotease *aprX* gene (*ΔaprX*), which showed only 20 % aphid mortality rate, till 68 h; 100 % aphid toxicity was only observed only after 80 h (Figure 6.7).

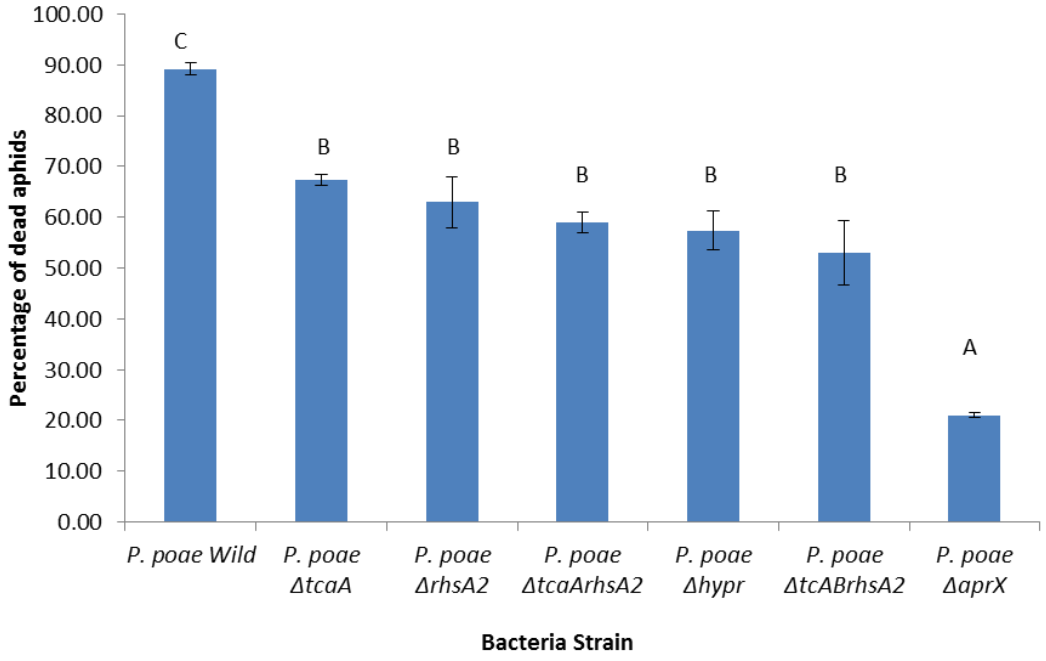


Figure 6.7: Assessment of aphid mortality by toxin deficient *P. poae* mutants. Mortality assay showing the percentage of dead aphids (N=10) at 48 h after ingestion of artificial diet inoculated with various bacterial strains (10^7 CFU mL^{-1}). Error bars represent the standard error of the mean of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD were shown as letters (where different letters on the graphs indicate statistically significant differences). (*P. poae ΔtcABrhsA2* – Tc-A (*tcaA* & *tcaB*), Tc-B (*tcaC*) and *rhsA2* genes deleted and *P. poae ΔtcaArhsA2* only tcaA gene with rhsA2 gene deleted)

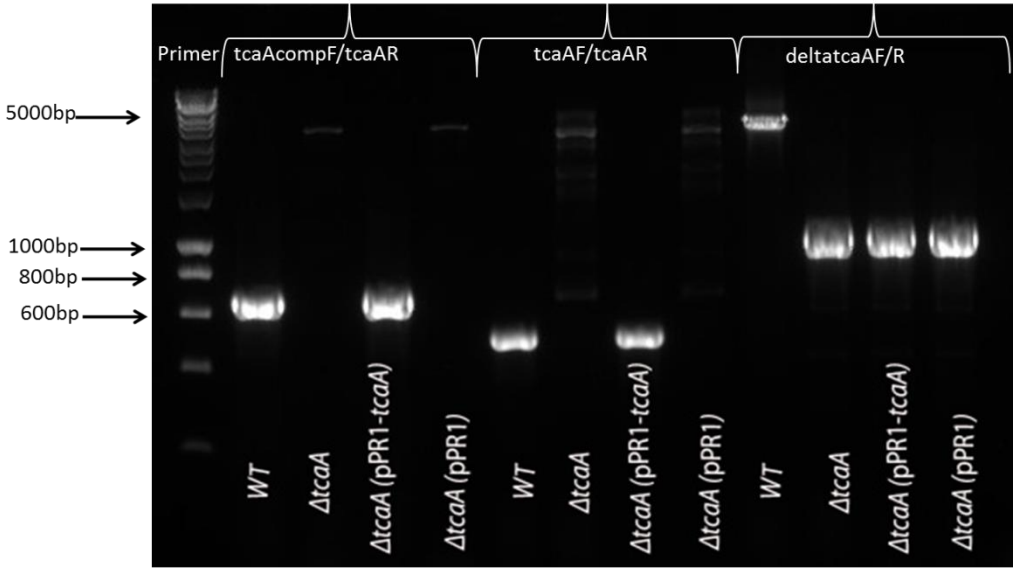
The effect of single mutation in potential toxin genes of *P. poae* indicated a decline in their pathogenicity with different rates. The *tcaA* component of Tc toxin deletion resulted in a reduction of toxicity to 65 % hence, deletion of A and B components of Tc toxin with other toxin genes might have produced a stronger effect on aphid mortality.

Since a complete loss of aphid killing ability was not observed, this suggested a multifactorial virulence phenotype existed. To test whether there were additive effects of toxin genes, I constructed double mutants to observe a complete reduction in aphid mortality through combinatorial effects of genes. I tried to construct all possible double mutants in a single toxin mutant background but this was only successful for two double mutants; Δ*tcaArhsA2* and knockout of the A and B subunits of *tc* gene cluster (genes *tcaA*, *tcaB* (Tc-A subunit) and *tcaC* (Tc-B subunit),Figure 6.2) and *rhsA2* (Δ*tcrhsA2*). The effect of these double mutants on aphid mortality displayed 56 % reduction in average toxicity, which was not significantly different from the single deletion effect of *tcaA* and *rhsA2* genes.

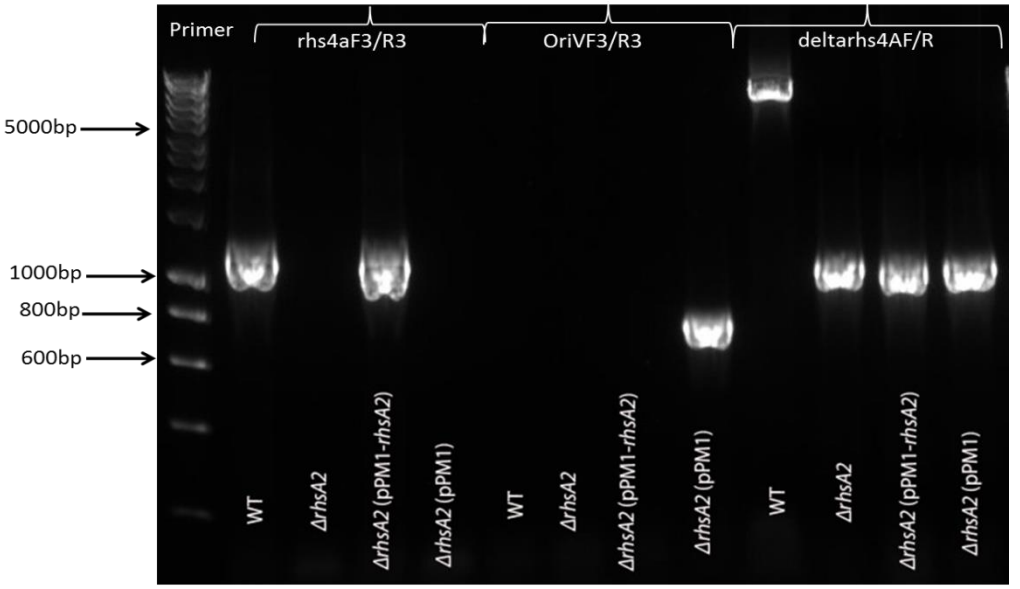
To verify my observations that the toxin genes play a role in pathogenesis, I cloned each toxin gene, under *lacZ* promoter, in pBBRMCS1-2 vector (except *rhsA2*, which could not be cloned into this vector and was thus cloned in pME6010) and transformed them to the

relevant mutant strain to enable expression and complementation; a vector only control was also made for each mutant.

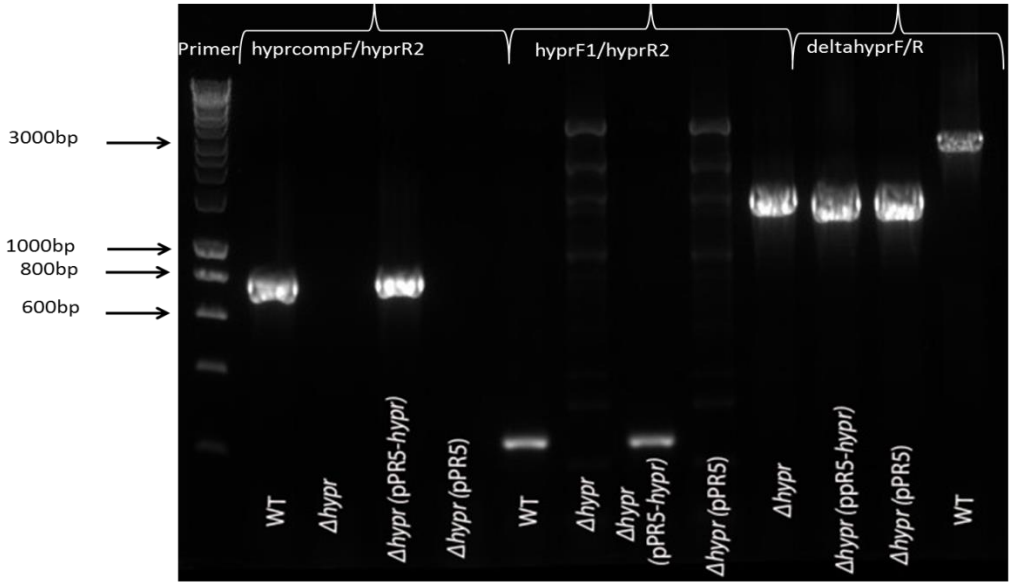
The presence of the complemented toxin gene in each mutant was confirmed by PCR, through the use of internal gene specific primers, vector primers and outer gene primer pairs. To generalise PCR products, toxin specific PCR products were only observed in wild type and complemented mutants and no band was observed in mutant and vector controls. The full length toxin gene PCR product was observed only in the wild type strain while the other strains only yielded deleted toxin gene PCR products (Figure 6.8A, 6.8B, 6.8C, 6.8D, 6.8E, 6.8F).


Primer
tcaAcompF/tcaAR
tcaAF/tcaAR
deltatcaAF/R
5000bp
1000bp
800bp
600bp
WT
ΔtcaA
ΔtcaA (pPR1-tcaA)
ΔtcaA (pPR1)
WT
ΔtcaA
ΔtcaA (pPR1-tcaA)
ΔtcaA (pPR1)
WT
ΔtcaA
ΔtcaA (pPR1-tcaA)
ΔtcaA (pPR1)

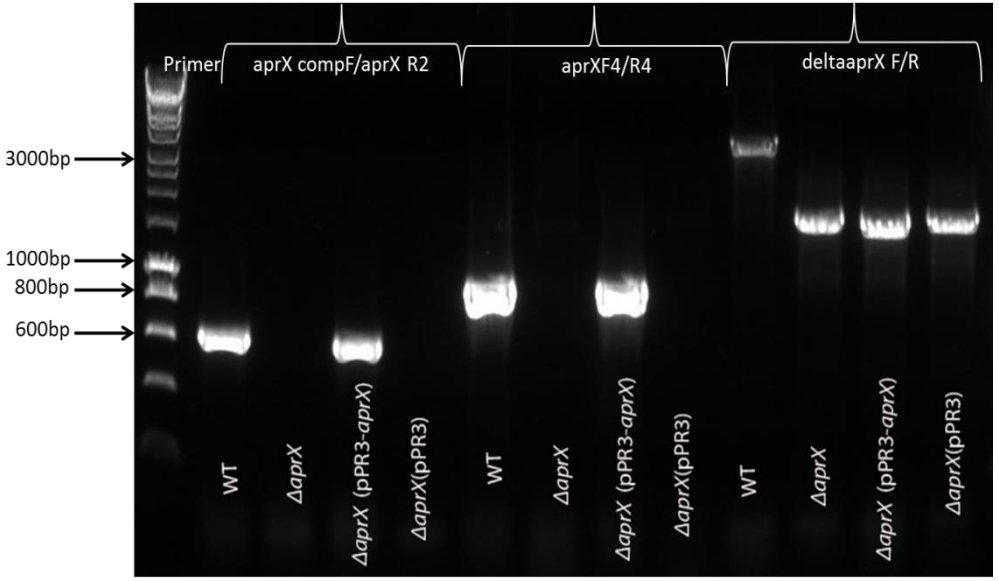
A


Primer
rhs4aF3/R3
OriVF3/R3
deltarhs4AF/R
5000bp
1000bp
800bp
600bp
WT
ΔrhsA2
ΔrhsA2 (pPM1-rhsA2)
ΔrhsA2 (pPM1)
WT
ΔrhsA2
ΔrhsA2 (pPM1-rhsA2)
ΔrhsA2 (pPM1)
WT
ΔrhsA2
ΔrhsA2 (pPM1-rhsA2)
ΔrhsA2 (pPM1)

B


Primer
hyprcompF/hyprR2
hyprF1/hyprR2
deltahyprF/R
3000bp
1000bp
800bp
600bp
WT
Δhypr
Δhypr (pPR5-hypr)
Δhypr (pPR5)
WT
Δhypr
Δhypr (pPR5-hypr)
Δhypr (pPR5)
Δhypr
Δhypr (ppR5-hypr)
Δhypr (pPR5)
WT

C


Primer
aprX compF/aprX R2
aprXF4/R4
deltaaprX F/R
3000bp
1000bp
800bp
600bp
WT
ΔaprX
ΔaprX (pPR3-aprX)
ΔaprX(pPR3)
WT
ΔaprX
ΔaprX (pPR3-aprX)
ΔaprX(pPR3)
WT
ΔaprX
ΔaprX (pPR3-aprX)
ΔaprX(pPR3)

D

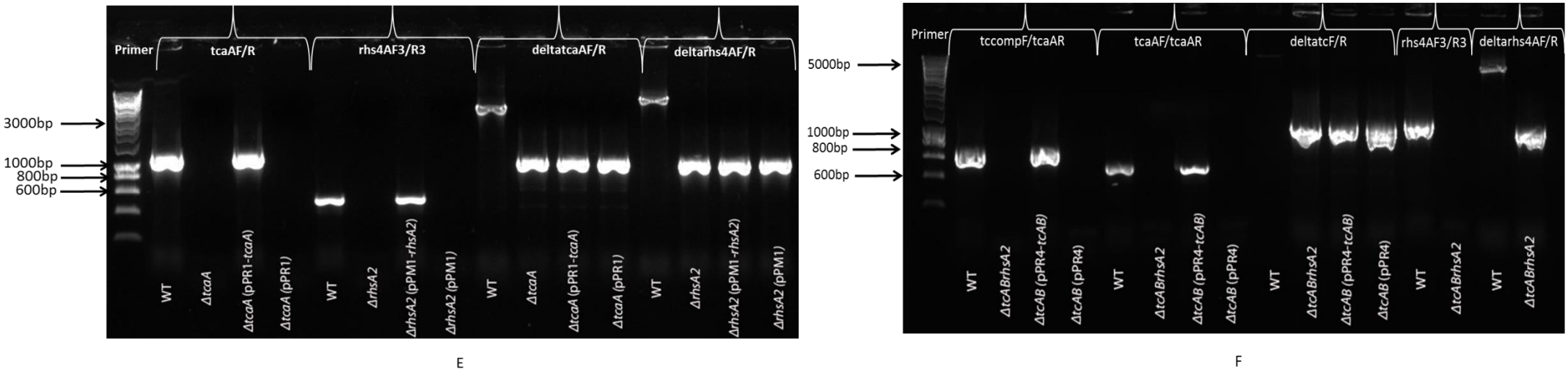


Figure 6.8 A-F: PCR amplifications of wild-type complemented and deleted regions for different toxin genes. Amplification of two first internal toxin primers showed presence of toxin gene in wild and complemented and no bands in vector and deleted ones. Last appropriate N_{ext}-C_{ext} primer pair used to amplify full length gene region in wild type and shorter toxin gene in rest of them. DNA size standard was loaded on the first lane of gel, with length in base pairs, indicated on the left.

A. *tcaA* gene – **Two inner sets of primers** (tcaAcompF/tcaAR & tcaAF/tcaAR) produced 800 & 656bp band respectively, in wild and complemented strains & no band in mutant & vector strains. **N_{ext}-C_{ext} primer pair** generated full length 3.3 kb in wild type and 1100 bp in rest of the strains.

B. *rhs4A* gene- **Two different set of primers** – **one gene specific** (rhs4aF3/R3) produced 1300bp, in wild and complemented strains & no band in mutant & vector strains. **Vector specific** OriVF3/R3 produced 700bp band only in vector control. **N_{ext}-C_{ext} primer pair** generated full length 5.2 kb in wild type and 1796 bp in rest of the strains.

C. *hypr* gene – **Two inner set of primers** (hyprcompF/hyprR & hyprF1/hyprR1) produced 920 & 232bp band respectively, in wild and complemented strains & no band in mutant & vector strains. **N_{ext}-C_{ext} primer pair** generated full length 2.8 kb in wild type and 1470 bp in rest of the strains.

D. *aprX* gene – **Two inner set of primers** (IPcompF/aprXR2 & aprxF4/R4) produced 978 & 663bp band respectively, in wild and complemented strains & no band in mutant & vector strains. N_{ext}-C_{ext} primer pair generated full length 3.1 kb in wild type and 1647 bp in rest of the strains.

E. *Both tcaA & rhsA2* – Similar inner sets of primers & external N_{ext}-C_{ext} primer pair for *tcaA* & *rhsA2* genes were used.

F. *Both tcAB & rhsA2* – Similar inner sets of primers & for *tcaA* & *rhsA2* genes were used. But external **N_{ext}-C_{ext} primer pair** generated full length 12 kb in wild type and 1200 bp in rest of the strains.

Toxicity assays were carried out on aphids with all the mutants, complemented mutants and vector control strains as well as with *P. poae* wild type. Aphid mortality readings were scored for 72 h. I observed that all complemented strains restored aphid toxicity similar to wild type levels and that they killed aphids within the same 48h time interval. The vector controls behaved like the mutants with taking longer to kill. The statistical test indicated no differences between wild type and all complemented strains; the same was seen in the comparison between all the mutants with their respective vector controls (Figure 6.9A, 6.9B, 6.9C, 6.9D, 6.9E).

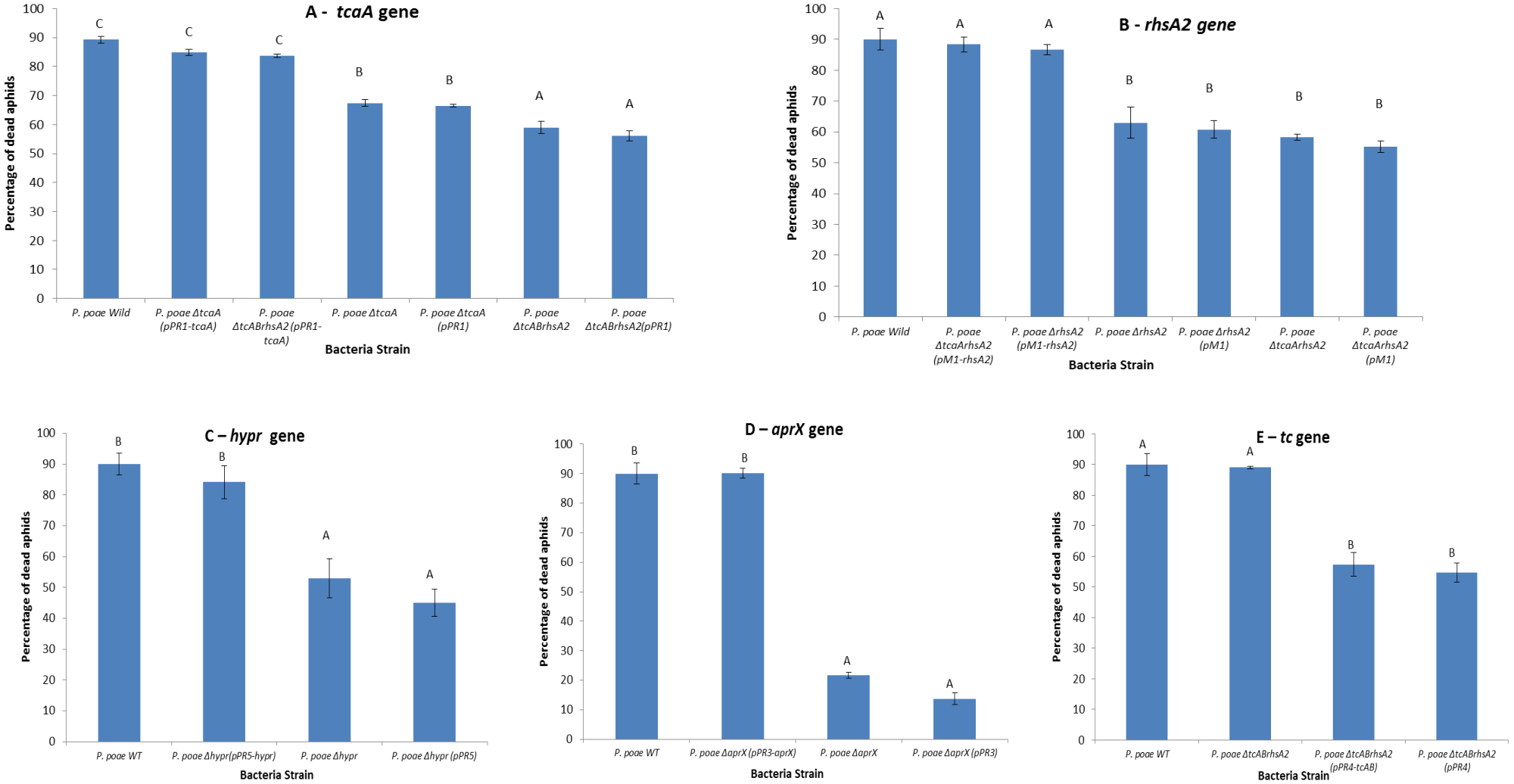


Figure 6.9: Assessment of aphid mortality by *P. poae* mutants complemented and vector strains. Mortality assay showing the percentage of dead aphids (N=10) at 48 h after ingestion of artificial diet inoculated with various bacterial strains (10^7 CFU mL^{-1}). Error bars represent standard error of the mean of three biological replicates. ANOVA detected statistically significant differences ($p<0.05$) and comparison of means by Tukey-Kramer HSD were shown as letters (where different letters on the graphs indicate statistically significant differences).

The delayed aphid killing time by mutants indicated that a growth defect might be linked to their pathogenesis. To assess growth fitness, I performed growth curves for all the mutants and wild type in different growth media (Mittler diet & LB media) at 20 ˚C (Figure 6.10). In both and LB medium, the lag phase was similar in *P. poae* wild type and all mutants with the exception of a longer lag phase for the *P. poae ΔaprX* mutant. To analyse any growth differences between mutants and wild type, the growth rate was calculated during the exponential phase of growth. In both media, the *P. poae ΔaprX* mutant exhibited a significantly lower Vmax value as compared to *P. poae* wildtype and other mutants. No significant differences in growth rate of the rest of mutants were observed (Table 6.12)

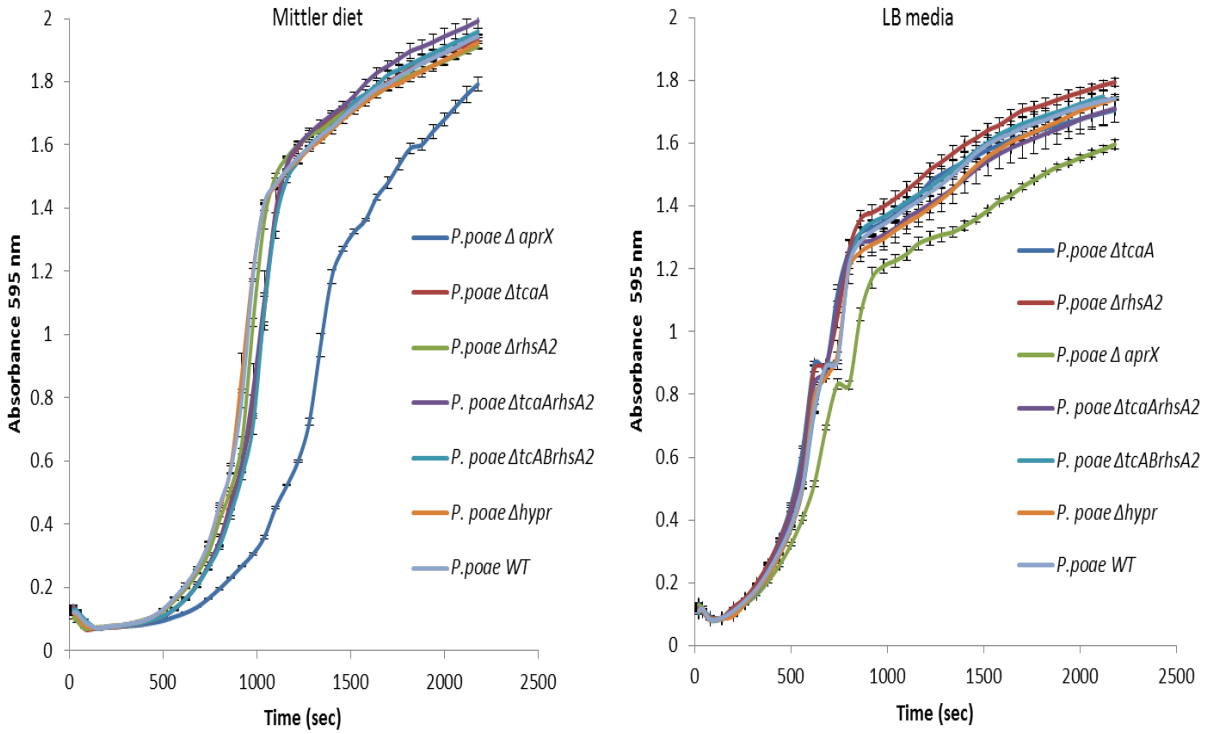


Figure 6.10: Growth curves of bacteria grown in different media. Bacteria were inoculated in to in a 96 well microtiter plate and grown for 24 h in a plate reader. The data presented are the mean and standard error of three biological replicates.

Table 6.12: Summary of *P. poae* wildtype and mutants growth performance in different growth media.			
Bacteria Strain	**Vmax (mOD min $^{-1}$)**	**Standard error**	**Level of significance (p=0.05)**
Growth rate in diet			
P. poae Wildtype	4.97	0.17	B
P. poae ΔaprX	2.92	0.24	A
P. poae ΔtcaA	4.50	0.32	B
P. poae ΔrhsA2	4.99	0.18	B
P. poae Δhypr	4.79	0.21	B
P. poae ΔtcaArhsA2	4.48	0.32	B
P. poae ΔtcABrhsA2	4.67	0.23	B
Growth rate in LB			
P. poae Wildtype	2.73	0.19	B
P. poae ΔaprX	2.00	0.18	A
P. poae ΔtcaA	2.71	0.09	B
P. poae ΔrhsA2	2.64	0.06	B
P. poae Δhypr	2.75	0.06	B
P. poae ΔtcaArhsA2	2.94	0.02	B
P. poae ΔtcABrhsA2	2.77	0.14	B

6.4 Discussion

The plant-beneficial pseudomonads are well known for their multiple behaviours and traits that enable them to not only survive and compete in the rhizosphere but also protect plants against fungal pathogens. The recent genomics work indicated that the genomes of certain soil-inhabiting and plant-associated members of the genus *Pseudomonas* carry specific loci possibly providing them with anti-insect activity (ffrench-Constant *et al.,* 2007). Recently, root-colonizing disease-suppressive agents *P. fluorescens* CHA0 and Pf-5, showed potent insecticidal activity, and were able to kill larvae of *G. mellonella*, *S. littoralis* and *Manduca sexta* within a short time span, at very low concentrations. The anti-insecticidal activity is linked to a genomic locus encoding a novel protein toxin that is related to the potent insect toxin Mcf1 of the entomopathogen *P. luminescens* (Péchy-Tarr *et al.,* 2008). This highlights the potential of plant associated pseudomonads to serve as a novel reservoir for biocontrol agents and microbial toxins with anti-insect activity.

Likewise, the current study demonstrated the oral insecticidal toxicity of a newly isolated rhizobacterium *P. poae*, to aphids both during *in vitro* as well as *in planta* assays. In order to understand *P. poae* virulence traits towards aphids, I employed genome sequence analysis and a gene mutagenesis approach.

6.4.1 *P. poae* Genome characteristics

The 6.1MB genome of *P. poae* genome sequencing revealed, 60 % GC content with 5,479 protein coding genes, showed similar findings of previously-sequenced genomes of *Pseudomonas* (Table 6.1). Moreover, the phylogenetic relationship with other pseudomonads based on seven core genes and housekeeping genes analysis indicated the closest fully sequenced relative, *P. fluorescens* SS10 (Figure 6.1 & Appendix Figure 8). It is interesting to note that both strains were isolated from different geographical areas, but from a similar ecosystem that is the rhizosphere of a crop. Both strains share similarities such as rhizosphere colonization, the presence of toxin complexes (Tc) genes, T3SS gene clusters (similar to *hrp*/*hrc* T3SS of the plant pathogen), siderophore biosynthetic genes (pyoverdine & hemophore) and chemotaxis genes (Table 6.2). These findings highlight the efficient spreading of toxin-complex gene homologs in bacteria typically regarded as non-insect interactors.

6.4.2 *P. poae* putative Virulence Factors Against Insects

An earlier study has demonstrated that *P. entomophila* virulence mainly relies on a number of potential virulence factors such as insecticidal toxins, proteases, putative haemolysins, hydrogen cyanide and novel secondary metabolites to infect and kill insects (Vodovar *et al.,* 2006).

In this current study, the *P. poae* genome was found to contain one toxin complex (Tc), two *Rhs* family protein, two alkaline proteases (*aprX, aprA*), hemolysins (exotoxins), and cell surface–associated virulence factors (hemagglutinin-like adhesins), several of which are implicated in toxicity to insects (Table 6.3 & 6.8). It has revealed pathways for biosynthesis of secondary metabolites such as siderophore (pyoverdine), haemophore, antibiotics (phenazine & mitomycin) that may contribute to biocontrol and virulence traits (Table 6.6). Moreover, chemotaxis, motility, and adhesion genes were found in *P. poae* which are required for colonization in a large range of habitats and extreme conditions (Table 6.9 & 6.10). Linking these to the RNA-Seq results, the altered *P. poae* gene profile revealed higher expression of iron acquisition genes (pyoverdine biosynthesis genes and siderophore uptake for iron transport), and haem acquisition system genes; all of these could be used to help the bacterium cope with iron restriction conditions inside aphids (Appendix Table 5A). The upregulation of urease and ammonia transporter genes could be used to combat low pH conditions inside insect gut Appendix Table 5D). I observed differential expression of *P. poae* oxidative stress genes (superoxide dismutases, catalase and alkyl hydrogen peroxide) which are involved in survival to low redox stress inside aphids (Appendix Table 5D). In addition to this, the differential expression of flagellar, adhesion, transcriptional regulators (more than 100) and membrane transporter genes suggests that *P. poae* is able to adapt to substantial substrate variations inside aphids (Appendix Table 5E,5F,5G).

All these *P. poae* virulence mechanisms against aphids are consistent with earlier findings of *P. entomophila* pathogenesis inside insects. However, *P. poae* is devoid of some secondary metabolite genes, encoding polyketides, pyoluteorin and 2, 4-diacetylphloroglucinol, pyrrolnitrin, and hydrogen cyanide production. Several of these have been implicated in *Caenorhabditis elegans* killing by *P. aeruginosa* and in the suppression of soil-borne plant pathogens by certain *Pseudomonas* species (Gallagher & Manoil, 2001; Haas & Defago, 2005).

The type three secretion system (T3SS) has been shown to have an important role in the pathogenicity of pseudomonads such as *P. aeruginosa* and *P. syringae,* pathogens of humans and plants, respectively. The T3SS is involved in cell-to-cell contact with the eukaryotic host and in bacterial virulence in these two pathogenic bacteria (Cornelis, 2006, 2010). Additionally, the Hrp and PSI-2 T3SSs play different roles in the life cycle of *P. stewartii* as it alternates between its insect vector and plant host. *P. stewartii* Hrc-Hrp T3SS, known to be essential for maize

pathogenesis, second T3SS (Pantoea secretion island 2 [PSI-2]) Inv-Mxi-Spa T3SS family, typically found in animal pathogens that is required for persistence in its flea beetle vector, *Chaetocnema pulicaria* (Melsh)(Correa *et al.,* 2012). Moreover, Type III secretion effectors systems are involved in blocking immune responses and phagocytosis or invasion and interfering with phagocyte maturation. *In vivo assays* of infection of the cutworm *Spodoptera littoralis* and the locust *Locusta migratoria* with a *P. luminescens* TT01 revealed *lopT* gene (TT3SS effector protein) was switched on only at sites of cellular defence reactions, such as nodulation, in insects (Brugirard-Ricaud *et al.,* 2005). Latter study showed the *Yersinia enterocolitica* T3SS intracellular acts as major role in survival/replication factor, with a secondary role to enhance invasion in a *Drosophila* S2 cells as a model system (Walker *et al.,* 2013).

In the current study, a type III secretion system (T3SS) gene cluster and three effector genes resembling genes related to the *hrc*/*hrp* T3SS and effectors of the plant pathogen *P. syringae* were found in the *P. poae* genome**.** The multiple forms of Type III secretion systems have also been found in strains of *P. fluorescens* (Preston *et al.,* 2001) thus it was not surprising to find a T3SS in *P. poae*, which is a member of the *P. fluorescens* species complex. The functional purpose of carrying the gene cluster is not clear, though there are some reports that the *P. fluorescens* T3SS may be used for fungus and oomycete interactions(Rezzonico *et al.,*2005; Cusano *et al.,* 2011).

Linking these to expression data, I observed downregulation of a few T3SS structural genes and no expression of T3SS effectors, regulatory and other genes, at the 38 h time point (Appendix Table 5H). Such a limited gene expression of T3SS genes at 38h post infection suggested it might not be important at that time point (perhaps it is expressed at an earlier time point). Therefore, a further time course of T3SS gene expression profiles should be conducted to shed some light on involvement of T3SS in virulence against aphids.

6.4.3 *P. poae* biocontrol capabilities

The genome annotation of *P. fluorescens* Pf-5 revealed secondary metabolites like phenazine, siderophores, pyoluteorin and 2,4-diacetylphloroglucinol, pyrrolnitrin have a direct role in the bacterium's capacity to suppress plant diseases. The bacterium also displays characteristics that contribute to epiphytic fitness on plant surfaces, such as iron acquisition and oxidative stress tolerance. The absence of known *P. syringae* phytotoxins, cellulases, pectinases, and pectin lyases, associated with degradation of plant cell walls and cell wall components, supports the conclusion that it has a commensal lifestyle on plants (Paulsen *et al.*, 2005). Likewise, my data indicated that the *P. poae* genome sequence encodes biosynthetic genes of secondary metabolites (phenazine and siderophores) and other commensal lifestyle traits (absence of

pectinases and phytotoxins) that may be required not only for interaction with insects but also for its lifestyle in plant surfaces, soil, aquatic or rhizosphere environments and its biocontrol capabilities.

A previous study showed 1-aminocyclopropanecarboxylate (ACC) deaminase catalyses the degradation of the ethylene precursor, ACC, into ammonium and α-ketobutyrate (2-oxobutanoic acid) and this has been linked to plant growth promotion activity in the rhizosphere (Glick *et al.,* 2007). In addition to this, Volatile organic compounds produced by rhizobacteria are involved in their interaction with plant-pathogenic microorganisms and host plants and show antimicrobial and plant-growth modulating activities (Vespermann *et al.,* 2007). I found genes for the biosynthesis of ACC deaminase and two volatile organic compounds (2,3-butanediol & acetoin) in the *P. poae* genome, which is consistent with the bacterium having potential plant promotion traits seen for many *Pseudomonas* species (Park *et al.,* 2015).

6.4.4 *P. poae* virulence revealed by a toxin gene mutagenesis

To directly identify factors that modulate the interaction between *P. poae* and *M. persicae*, I used a Tn*5*-derived mutant library of *P. poae,* which was prepared in Livermore PhD project and screened 768 individuals for their aphid toxicity. The screening did not identify any mutants with altered aphid toxicity compared with the *P. poae* wildtype strain. Jacobs *et al.* described that bioinformatic comparison and statistical analysis of 30,100 mutants showed that nearly 90 % of the open reading frames (ORF= 5,200) in the *P. aeruginosa* PAO1 genome (6.1 Mb) had been disrupted at once (Jacobs *et al.,* 2003). Similarly in the current study, based on above estimates, 2000 mutants in the total transposon library was not enough to predict the insertion in 5,699 ORFs of *P. poae*. Such a small estimate of 768 mutants screening was far from saturation range therefore in future a much larger number of random mutants would be required to predict insertions and identify mutants involved in pathogenesis. However, a targeted knockout mutagenesis of selected toxin genes (*tc, tcaA, rhsA2, aprX, hypr*) did reveal significant decreases in aphid mortality.

Toxin complex (Tc) genes that encode insect toxins are found in many *Pseudomonas* sp. (ffrench-Constant *et al.,* 2007). Although the exact mode of action of these orally active toxins is still not fully resolved. One study has demonstrated that oral ingestion of the purified recombinant C component of the toxin complex TccC-like protein from *P. taiwanensis* caused high mortality in Drosophila (Liu *et al.,* 2010). My data showed that *tcaA*, *rhsA2,* and *hypr* mutants have lower toxicity toward *M. persicae* than the wildtype. However, all three mutants still induce a 68 %, 62 % and 50 % mortality rate, respectively, indicating these were not the sole causes of aphid killing

and perhaps indicating either relatedness in the determining the final product (e.g. proximity of *hypr* to *tcaA* may indicate they are part of the same toxin production system) or redundancy.

Two double mutants were also engineered: the *ΔtcABrhsA2* strain had the two sunbunits of tc toxin gene cluster, including all *tcaA*, *tcaB* (Tc-A subunit) and *tcaC* (Tc-B subunit) components, removed along with the *rhsA2* toxin; and in the case of *ΔtcaArhsA2* mutant, only *tcaA* and *rhsA2* deleted. Mutants (Δ*tcaArhsA2* and Δ*tcABrhsA2*), showed further reduction in aphid mortality to 59 % and 57 % respectively, but these were not significant from the single mutants. The effect of double toxin mutants on aphid mortality reflected similar reports of insertional mutagenesis of one and two gene-combinations of *S. entomophila* toxic determinants: "*sepA*" (similar to *tcaA*), "*sepB*" (similar to *tcaC*) "*sepC*" (similar to *rhsA2*) were responsible for the reduction of gut toxicity and cessation of feeding in *Costelytra zealandica.* Cessation of feeding was only completely abolished in the insect when all toxicity determinants were mutagenized (Hurst *et al.,* 2000). Similarly, both double mutants confirmed the partial role of *tcaA* gene in the aphid toxicity, which was consistent to similar reports of heterologous expression of *Photorhabdus* W14 TcdA (which is homologous to *P. poae tcaA* and *tcaB*), which were not sufficient to produce maximum toxicity against *M. sexta* (Waterfield *et al.,* 2005). Previous research described that increased activity of the toxin TcdA1 requires potentiation by two gene products of TcaC (TcdB1) and TccC2 and showed these same pairs can also cross-potentiate a second toxin, TcaA1B1 (Waterfield *et al.,* 2005). Another study also demonstrated that Tc-A (*tcaA, tcaB*) act as main functional unit of tc toxin that induces the actin clustering molecular mechanism of death in target cells through action of tc subunits {Tc-B (tcaC) &Tc-C(tccC)}, which function as a molecular syringe allowing membrane translocation of the functional Tc component (Tc-A) (Meusch *et al.,* 2014)

In my study, I examined aphid toxicity with mutants of Tc-A (*tcaA*, *tcaB*) Tc-B (*tcaC*) subunits of *tc* gene with combination of *rhsA2* genetic element. To understand the full mechanism of Tc toxin against aphids, a further gene deletion of Tc-C subunit (*P. poae tccC2* gene) with combination of other subunits of Tc toxin would be required to be investigated.

I also observed a hypothetical protein upstream of the Tc toxin open reading frame, which may be linked to the *tc* gene cluster, perhaps as a regulator which is often found flanking operons. Additionally, upregulation of *hypr* genes were reported in treated bacteria transcript profiles in both RNA-Seq and qRT-PCR results. Thus, it is predicted that loss of this putative regulator (*hypr* gene) leads to reduction in toxicity against aphids due to down regulation of the *tc* genes. These observations are consistent to earlier findings which state that hypothetical proteins around *tc* genes are potentially involved in virulence (Crennell *et al.,* 2000; Waterfield *et al.,* 2001).

Another single deletion mutant of metalloprotease *aprX* caused a significant drop in aphid toxicity to 20 %. These results are consistent with a previous report where, a metalloprotease mutant of *P. entomophila* (*aprA* mutant) was shown to be slightly less virulent and had a reduced persistence in *D. melanogaster* (Vodovar *et al.,* 2005).

The toxin mutagenesis findings suggested that all toxins play a role in *P. poae* virulence by reducing the aphid mortality at different rates. To verify this assumption, I constructed a complementary strain of all toxin mutants. With these complementary strains I tested the importance of traits according to the postulates of Koch (Koch, 1882; Falkow, 1988), which says that if a specific factor is eliminated, its effect will disappear. If, when this factor is restored, the effect is observed again, then this factor was undoubtedly the cause of this effect. So if this complemented strain would regain full toxicity towards *M. persicae*, it would confirm that the actual toxin is of importance in the virulence of *P. poae*. I have tested the vector alone in all the toxin mutants to testify any effects on aphid mortality were due to the cloned gene. All complemented strains of single and double mutants expressed their toxin genes under *lacZ* promoter and restored full aphid mortality as similar to wild type strain. Additionally, vector strains also displayed similar mutant aphid mortality rate which depicted toxicity was not affected by presence of expression vector.

Moreover, growth curve analyses showed similar growth rate (Vmax) between all mutants and wild type except for the *aprX* mutant. The lower growth rate (Vmax) of *ΔaprX* mutant as compared to wild type might suggest longer killing time i.e. 80 h required to show full toxicity.

A previous study described *P. fluorescens* ON2 exploits protein sources in the environment by constitutive expression of AprX proteinase during growth unless a preferred carbon source such as citrate or other inhibitors are present in its environment. It reported slower growth of an *aprX* mutant than wild type when growth medium was supplemented by casein and other protein sources, indicating a protease deficient strain could not utilize added protein for growth (Nicolaisen *et al.,* 2012).

Correspondingly in my study, reduced growth rate of the *aprX* mutant (protease deficient strain) might suggest inability to utilize protein sources of both media in Mittler diet and LB media. Further investigation of the growth kinetics of the *aprX* mutant in minimal medium supplemented with different carbon and nitrogen sources would shed some light on nutrient auxotrophy of the *aprX* mutant strain. The study described the contiguous genes *aprX-inh-aprDEF-prtAB-lipA* function as an operon, transcribed from the *aprX* promoter. The data showed insertion mutation in the *aprX* promoter had complete absence of both proteolytic and lipase activity. Such mutation of *aprX* could have a polar effect on the expression *of* lipA, consistent with the *aprX-lipA* region

constituting an operon (Woods *et al.*, 2001). Likewise, future work would be conducted to envisage any potential effects on transcription of other genes (growth and metabolism) by an *aprX* gene mutation. This would help in identifying extra potential targets of host-bacteria interaction during pathogenesis.

Previous work has observed the role of A24tox toxin of *Xenorhabdus nematophila* secreted in the growth phase II state and when this toxin is injected into larvae at doses of around 30–40 ng g^{-1}, it caused death in *Galleria mellonella* and *Helicoverpa armigera* (Brown *et al.,* 2004). Although I was not successful in identifying the secreted toxin of *P. poae* in filtrates of Mittler diet and LB media at different time points, the preliminary total protein quantification of Mittler diet filtrates at different time points showed increasing protein amount from 12 h to 72 h with no change of protein blank filtrate. These results confirmed the presence of secreted proteins accumulating in the growth medium of *P. poae* cells, but may indicate the proteins are not in an active conformation or are rapidly degraded outside the aphid. This should be tested in the future with improved methodology coupled with identification of secreted proteins by mass spectrometry.

Taken together, it is hypothesized that all toxins worked together which produce additive effects on aphid toxicity. Thus, future investigation of virulence mechanisms would be carried out by creating double or triple mutants with combination of *aprX and rhsA2 or tc* genes. Also, expression analysis at different infection time points may help to understand if different genes, including toxins and type III secretion system, are expressed at different points in the infection cycle. Finally, the complete genome sequence of *P. poae* provides a framework for further studies to characterize its pathogenic properties and for a host-pathogen system in which both organisms are amenable to genetic and genomic analysis.

7 General Discussion

The work described in this PhD has revealed how novel plant-associated bacteria may have application as alternative means to control economically important aphid crop pests. I have carried out a range of fundamental experiments to characterise the efficacy of aphid killing by the different bacteria, including characterising the lethal concentration for 50 % of aphid death (Chapter 3). To characterise the mechanism of aphid killing, I focussed on the most pathogenic bacterium, *Pseudomonas poae* for in depth analysis. I further examined the dynamics of the interaction including uptake of bacteria from surfaces and from within plant tissues, bacterial population growth inside the aphid and altered aphid behaviour towards bacterial-treated plants (Chapter 4). Both transcriptomic and genomic analyses identified potential targets in *P. poae* that could be involved in pathogenesis against aphids (Chapter 5 & 6).

7.1 Dose–response relationships for aphid toxicity

The present work demonstrated for the first time bacteria susceptibility differences in insecticide resistant clones (differing in their armoury of insecticide-resistance mechanisms) and evaluated any fitness cost penalties associated with the known resistance mechanisms. In initial screening, I identified six plant derived bacteria which caused 50-100 % mortality to various kinds of insecticide resistant clones.

To learn more about the insecticidal potency of all bacteria, dose-response assays were performed by ingestion of bacterial inocula ranging from 100 to 10^7 CFU mL^{-1} washed cells in Mittler diet and insect mortality was recorded over a period of 72 h (Figure 3.4 A-G). Based on the data obtained, sub-lethal doses of bacteria were identified for all aphid clones and significant variation in susceptibility was observed. Each insecticide resistant clone carries two or more resistance mechanisms (enhanced production of detoxification enzymes or altered insecticide target sites) which are expected to reduce fitness in the absence of insecticide selection pressure. However, I didn't identify any consistent lower bacteria susceptibility in resistant clones after challenge of all tested bacteria. As a result, no generalised trend between resistance status of aphid clone and susceptibility to all bacterial species has been identified.

Nevertheless, *P. poae* bacterium was reported as more pathogenic to all three insecticide resistant clones with the most resistant aphid clone (5444B) an exception to this rule. These results suggested *P. poae* is more toxic to resistant clones than susceptible clones. Additionally, inoculation dose as few as 100 cells of *P. poae* in diet were sufficient to cause 100 % mortality within 85 h post infection. At higher doses, 100 % mortality was reached after even shorter

incubation times (Figure 3.3 & 3.4). Thus, *P. poae* exhibited considerable toxicity to a range of insecticide resistant aphid clones even at the lower inoculation doses in Mittler diet.

Recent studies have been reported that metabolic detoxification mechanisms in insects are energetically expensive, which would result in an allocation trade-off between defence mechanisms and other biological functions such as growth and reproduction (Karban & Agrawal, 2002; Nielsen *et al.,* 2006; Manson & Thomson, 2009). Certainly in this work, such trade off may make the 794J2, New green and 5191A resistant clones more susceptible to *P. poae* attack.

It will be necessary to screen a greater number of insecticide resistant and susceptible aphid clones to fully assess if fitness costs associated with insecticide resistance enhance the susceptibility of aphids to *P. poae*. Nevertheless there was no evidence of strong cross-resistance between insecticides and *P. poae*, indicating this biocontrol is insecticide resistance breaking. The significance and implications of this are discussed in section resistance management strategies.

To predict the robustness of *P. poae* control to evolve bacterial resistance it is useful to understand the aphid killing mechanism of *P. poae* to inform future pest and resistance management strategies.

7.2 *P. poae* pathogenesis in aphids

An analysis of the transcriptome of *P. poae* infecting aphids was performed to better understand the mechanisms of pathogenicity of the bacterium towards insects and in a reciprocal analysis of gene expression in aphids how the host responds to the infection.

The aphid gut is a continuous tube that runs from the mouth to the anus. The ectodermal origin cuticle lining cover both the foregut and hindgut region of the alimentary canal. The midgut region comprises an endodermal peritrophic membrane which forms a barrier between the epithelial layer and the midgut lumen, to accommodate the food bolus. The distribution of pH values and presence of various hydrolytic enzymes along the aphid gut lining can inhibit growth or kill ingested microorganisms (Cristofoletti *et al.*, 2003). In the current study, the uptake of ingested bacteria with food is assumed to pass through the mouth and then be passively transferred to the distal end of the foregut, and then the midgut, by peristaltic contractions.

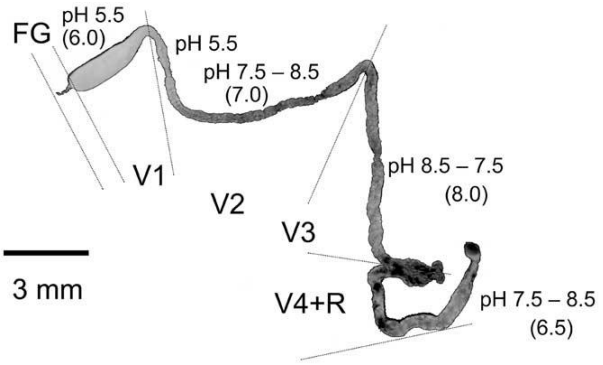


Figure 7.1: pH of gut contents at different sites in *A. pisum*. Ranges correspond to determinations performed on guts coloured by a universal pH indicator. Parentheses refer to averages of at least four determinations (reproducible within 0.2 pH units) carried out in isolated gut contents. FG, foregut; V1–V4 are sections of the ventriculus; R, rectum. Aphids have no Malpighian tubules. Figure designed and published by Cristofoletti *et al.,* 2003.

Previous work describing oral uptake of *Dickeya dadantii* by pea aphid, observed that bacteria start multiplying in the gut as early as one day post-infection; they crossed the gut epithelium and invaded the body cavity of the insect, with a preferential localization in the fat body (Costechareyre *et al.,* 2013). This infection continues to spread in other organs, such as the brain or the embryos, and it is probable that death is provoked by septicaemia. Such septicemia led to insect death when the bacterial load reached about 10^8 CFU mL^{-1}.

Likewise, in my study the most probable reason of infection through oral ingestion is bacterial multiplication in the insect gut. The *P. poae* enumeration from infected aphids showed an increase in *P. poae* population until 48 h with no bacterial counts from control aphids (Figure 6.6). The rapid multiplication of *P. poae* inside aphids suggested a similar colonization strategy as seen with *D. dadantii* inside host tissues. To visualize the localization of *P. poae* inside the aphid host, I have used different GFP, CFP & RFP *E.coli* construct strains to tag *P. poae* with fluorescent protein. However, although the genes could be moved into *P. poae*, expression of the fluorescent protein was not observed therefore confounding localization of *P. poae* inside *M. persicae*.

The bacterial presence in the gut will lead to it facing the insect immune response and that will necessitate an ability to counteract the host defence. The first line of defence at the site of infection may include measures such as antimicrobial peptide (AMP) production and lysozyme (digestive enzymes) secretion in the gut epithelium; this has previously been observed to be triggered by *E. c. carotovora*, *P. entomophila* and *S. marcescens* (Basset *et al.,* 2003*;* Vodovar *et al.,* 2005*;* Nehme *et al.*, 2007).Cathepsin-L proteases are predominantly found in the midgut of aphids and other insect species where they function as digestive enzymes, tissue remodelling during insect metamorphosis and involved in immunological processes (Gaget *et al.*, 2014; Wang *et al.,* 2010). The latter study indicated the role of the lysosomal system in *Buchnera* degradation

where cathepsin L proteases could participate in the regulation of symbiont populations inside bacteriocytes (Nishikori *et al.,* 2009). These recent observation suggests symbiotic and pathogenic bacteria have to avoid lysosomal degradation in order to establish an interaction. The signalling pathways such as IMD and JNK play a crucial role against Gram-negative bacteria infection in *Drosophila* and other insects (Christophides *et al.,* 2004; Evans *et al.,* 2006; Lemaitre & Hoffmann, 2007).

However in pea aphid, major bacteria effectors of the IMD signalling pathway - antimicrobial peptides (AMP) and genes involved in the IMD pathway (including peptidoglycan recognition proteins (PGRPs) and the central IMD protein) are absent but it has orthologs for most components of the JNK pathway , which plays a role in humoral immune response (Gerardo *et al.,* 2010). A previous study characterized the response of *A. pisum* to the ingestion of the free-living *S. symbiotica CWBI-2.3T* in comparison to the ingestion of the pathogenic *Serratia marcescens* Db11 at the early steps in the infection process (17 h post infection). No immune response was triggered by *S. symbiotica* and it colonized the gut within a few days. However, infection of *S. marcescens* Db11 caused a moderate host immune response (activation of host lysosome machinery and JNK pathway) supporting the hypothesis of a finely-tuned immune response set-up for fighting pathogens while avoiding harm to mutualistic partners (Renoz *et al.*, 2015).

In my study, only a few *M. persicae* saliva and gut specific counter mechanisms were induced after ingestion of *P. poae*. This included induction of aphid saliva takeout gene and other digestion genes (glucosidase, mucin and lipases) suggesting their role in actively degrading bacterial cell components (Appendix Table 3B). The elevation of two salivary apoptotic genes and suppression of various drug metabolism genes (esterases, Cytochrome b561 and heat shock proteins) in aphids occurs as a consequence of *P. poae* infection (Appendix Table 3A). The differential expression of aphid proteases (including cathespin, aminopeptidase N-like & venom protease) may function to digest bacteria and represent a mechanism by which the insect host would control invasive bacteria population (Appendix Table 3B). Moreover, I observed no change in expression of innate immune motifs such as **D**own **s**yndrome **c**ell **a**dhesion **m**olecule (Dscam2), members of the **I**nhibitor of **AP**optosis (IAP) and other JNK signalling molecules for aphids infected by *P. poae* 38 h post-ingestion in comparison to the control although these might be expressed at an earlier stage of infection (Appendix Table 4A). The absence of response for several immune genes and the weak defence response observed in my study is not as surprising as the aphid immune system showed limited response in comparison to other well-studied insects, such as flies, mosquitoes, beetles and wasps (Altincicek *et al.,* 2011; Gerardo *et al.,* 2010). It is also hypothesized that aphid symbiont interactions may have led to the loss of immune pathways to facilitate long term association with symbiotic bacteria (Dubreuil *et al.,* 2014). This reduced

immune response in aphids after *P. poae* infection supports the hypothesis of adjustment of the host immune response for the maintenance of symbionts. However, further study is needed to understand the biological role of cathepsin and other target immune genes in the regulation of *P. poae* and symbiont populations in aphids.

The *P. poae* transcriptomic data revealed induction of a large number of genes, many of which are defence and stress gene machinery that are likely employed to counteract the insect response. An analysis of pathogenesis in different bacteria indicated the presence of persistence factors (such as Evf, Afp, Ymt and Hms) and factors that counteract the immune response (such as AprA, cytolysins and haemocyte killing factors) (Haas & Defago, 2005). Moreover, all these toxic factors may act together for the full virulence phenotype. Likewise, I only observed higher expression of the *aprX* protease gene with other toxin genes such as *aprA*, *tcaC* subunit of Tc and *Rhs* reduced in expression (Appendix Table 5D). The downregulation of most of the toxin genes suggested they are not expressed at the late infection phase assessed, perhaps indicating they are expressed at an early phase of infection to help evade the host immune response. I was unable to assess earlier time points as the 38h time point was selected in order to ensure sufficient increase in bacterial load and hence detection of bacterial transcripts in RNA-seq. Many degradative enzymes, including lipases, proteases and haemolysins, might also contribute to the virulence of entomopathogens. The differential expression of proteases (*aprA* & *aprX)* in my study suggested, in addition to degrading free proteins, these enzymes might be involved in the destruction of cells and tissues to facilitate colonization of the insect body. Moreover, In addition to protein factors, several entomopathogens produce toxic secondary metabolites that impaired host machinery or compete with beneficial microbes (Duchaud *et al.,* 2003; Vodovar *et al.,* 2006). The *P. poae* altered gene profiles showed induction of secondary metabolite biosynthetic genes (phenazine and pyoverdine synthesis), iron uptake, oxidative stress and additional virulence genes which might have a combinatorial action in pathogenesis.

7.3 Gene mutagenesis in *P. poae*

Targeted toxin gene mutagenesis in this study resulted in the reduction of aphid mortality in my *in vitro assay* suggesting their role in pathogenesis. In *Salmonella*, the VRP1 domain of SpvA protein (similar to tcaA component of Tc-A subunit) and ADP-ribosyltransferase domain of SpvB toxin protein (*tcaC* component of Tc-B subunit) has been involved in depolymerisation of actin, destruction of the cytoskeleton and cytotoxicity (Boyd *et al.*, 1998; Gotoh *et al.,* 2003;). In my study, the reduction of aphid mortality to 59 % and 52 % after the deletion of *tcaA* and both Tc-A and Tc-B subunits suggested the similar role of VRP1 domain of TcaA protein and SpvB domain of

TcaC protein in actin condensation that induce cell dysfunctions in the aphid gut including increased cell membrane permeability and disruption of cell junction rearrangements (Figure 6.7).

The YD repeat/RHS element found in the *Pantoea stewartii* yo**u** **c**annot **p**ass (Ucp) protein enables the bacteria to colonize in the gut of the pea aphid and form aggregates, which finally reduced honeydew excretion and caused death (Stavrinides *et al.,* 2010). Similarly, the above stated interaction of bacteria rhs like gene inside the insect gut supported the role of *P. poae* RhsA2 toxin in the aphid pathogenesis.

Additionally, the genome annotation of *P. poae* revealed another YD repeat in the TccC2 component of Tc-C subunit (Table 6.3). It has been demonstrated that full toxicity of Tc toxin protein reconstituted by the TcdA-like component or TcaAB (similar to TcaA and TcaB) is potentiated by both TcdB-like/TcaC-like and TccC-like components. The work also revealed that both TcdB-like/TcaC-like and TccC-like components should be expressed in the same cytoplasm to generate a productive interaction (Waterfield *et al.,* 2005). The latter study recognized a novel export system governed by TcB and TcC subunits to facilitate secretion of the larger TcA-subunit (Yang & Waterfield, 2013). Hence, the further mutagenesis study with *tccC and tcaC* gene combination would enable us to understand the full role of Tc toxin in the aphid pathogenesis.

The *aprX* gene deletion resulted in a significant decline in aphid toxicity by 80 %, which suggested that this extracellular protease might facilitate bacterial colonization by inducing damage of host tissue and actively suppressing the immune response (Figure 6.7). A previous study showed the role of two proteases, alkaline protease AprA and the elastase LasB, of *P. aeruginosa* which are able to degrade exogenous flagellin and prevents flagellin-mediated immune recognition inside the insect host (Casilag *et al.,* 2016). Further work is needed to elucidate the role of *P. poae* AprA and AprX proteases in subverting aphid immune responses.

Moreover, another recent study demonstrated that there is no strong correlation between genome wide expression and knockout fitness to examine bacteria metabolism, virulence, and physiology during infection (Whiteley *et al*., 2014). They suggested genetic redundancy may cover the function of other genes in fitness mostly in studies of single mutant strains and problems of cross-complementation, which affects the mutant phenotypes (Whiteley *et al.,* 2014).

Likewise, in this study, gene deletion was carried out on a few toxin genes and further linking to RNA Seq data showed a positive correlation with them. However, to explore a stronger relationship between genetic mutagenesis and altered *P. poae* expression data, a number of genes under different categories need to be investigated.

7.4 Potential use of *P. poae* in Integrated Pest Management

Microbe-based pesticides are more likely to be preferred in Integrated Pest Management (IPM) strategies due to their mode of action, which is normally more complex than conventional chemicals, targeting a diversity of action sites which reduces the chances of resistance development. Although microbial pesticides or their products can be utilised alone for pest management, their use in rotation or combination with synthetic chemistry or other means of control is strongly encouraged to achieve full efficacy and longer-term sustainability. Many studies emphasized compatibility and synergistic effects of entomopathogenic bacteria and chemical substances (Morris, 1972; Seleena *et al.,* 1999; Musser *et al.,* 2006). *Pseudomonas*-based formulations are considered challenging for deployment because survival of microorganisms during the manufacturing process and long-term storage has the potential to reduce their efficacy. Furthermore, there are reports of inconsistency in field trials, and biosafety concerns due to application of opportunistic human pathogens such as *P. aeruginosa* (Walsh *et al.,* 2001).

Hence, during formulations of these microorganisms, all quality assurance and efficacy tests along with extensive study of their effects on plants and insect are required. This would expand application methods by amending the formulation strategy. The Comprehensive risk analysis of novel bacterial strain application is essential to ensure that a bacterium does not cause any detrimental or toxic effects on human health and environment safety.

As illustrated in the earlier chapters of this study, the plant derived *P. poae* possess multiple genes that are beneficial to the plant in terms of growth and protection against various pests. These include insect pathogenicity, antagonism to soil borne pathogens and plant growth promotion. During formulation of novel bacterial strains as plant protection products, the efficacy of the bacterium as an insecticidal organism, the persistence and competition on plant surfaces, and the resistance development should be considered. In my study, *P. poae* displayed oral insecticidal activity in feeding assays with artificial diet and also when applied as a foliar spray to control aphid infestation on different crop plants by (55 % efficacy rate) (Figure 4.4). Furthermore, Livermore (2016) showed plant growth promotion effects in pepper plants when the plants were irrigated with different concentrations of *P. poae*. An increase in dry weight and height of leaf and stem as well as root length for *P. poae*-treated pepper plants as compared to water sprayed plants were reported (Livermore, 2016). In the same study no immune response (such as melanisation) was observed in *G. mellonella* (model organism for *in vivo* toxicology, proxy for human immunity) after injection of *P. poae.* Additionally, *P. poae* did not grow on royal jelly or larvae of bees suggesting no adverse effect on this natural pollinator (Livermore, 2016). No hypersensitive response (HR) was observed on tested plant species over four weeks after foliar

spray and infiltration of *P. poae* (Figure 4.3). These data provide promise that *P. poae* is environmentally friendly and probably harmless to humans, which would make it an ideal target for use in IPM strategies. All the above results showed consistency with earlier findings of root colonizing biocontrol strains, like *P. protegens* and *P. chlororaphis,* which displayed potent oral insecticidal activity and plant promoting traits (Ruffner, 2013; Flury *et al.,* 2016).

The previous work described the role of naturally occurring bacteria found in the gut or environment of a targeted insect applied as inundative releases particularly in soil habitats to provide short-term pest control. The persistence and recycling of bacterium and subsequent use of bacterial infective agents (toxins and other detrimental substances) within the gut of targeted insects would be effective to control infestation (Lacey *et al.,* 2007). Many *P. fluorescens* strains have an ability to colonize in different niches and show environmental persistence (Hirano & Upper, 2000; Vodovar *et al.,* 2005; Loper *et al.*, 2012; Viggor *et al.,* 2013). These microbes can be applied on plants either as a seed treatment, soil drench, or foliar spray. Therefore, rhizosphere competent Pseudomonads are considered as ideal candidates and applied as inoculations for long-term control before pest insects pose a problem to the plant population.

For effective delivery of microbial inoculants, commercial seed coating and pelleting procedures have been employed. Isolates of *B. megaterium*, *A. histidonolovorans* and *P. fluorescens* have survived in high numbers and been able to colonize in peat in sealed gas-permeable bags, which suggested peat had potential carrier material for incorporating the isolates into the commercial pelleting process. However, all of the pelleted preparations using the peat-based formulations performed poorly in terms of survival of the isolate when stored at room temperature (Walker *et al.*, 2002). Another study described a comparative account of seed soaking, encapsulation in alginate, pelleting using an inoculated peat carrier or seed priming methods for application of *Pseudomonas marginalis/P. putida* P1W1 to sugar-beet seed. Over all the other application strategies tested, priming inoculation displayed significantly greater viable bacteria populations on pelleted seed. After pelleting with fungicides and drying at 40 $^{\circ}$C, bacteria maintained populations of >6.6 $\log_{10}$ CFU g^{-1} seed during 4 months storage at ambient temperature with little loss in viability (Walker *et al.,* 2004).

Additionally, biotechnology can be applied to further improve the biocontrol efficacy of these strains. This involves creating transgenic strains that combine multiple mechanisms of action. In the literature, many studies described genetic modifications via modulating function of specific transcriptional activators /repressor or post transcriptional regulators to improve efficacy of biocontrol strains. For example, overexpression of *rpoD*, which encodes the housekeeping sigma factor σ^{70}, resulted in increased production of PLT and DAPG of *P. fluorescens* CHAO and improved its biocontrol efficacy (Maurhofer *et al.,* 1992) . The knockout of repressor gene *phlF and*

overexpression of the *phlA–D* operon of *P. fluorescens* resulted in Phl overproduction and, concurrently, improved biocontrol efficacy against *P. ultimum* in a laboratory microcosm trial (Delany *et al.,* 2000; Delany *et al.,* 2001). Another study showed post-transcriptional control mechanisms such as GacS/GacA (environmental sensor kinase and response regulator of a two-component system) could be employed to overexpress secondary metabolite production (Aarons *et al.,* 2000; Haas & Keel, 2003).

In my study, foliar spray of *P. poae* displayed a good efficacy rate ~ 55 % in an *in planta* assay which may be improved in future through application of different strategies described in Figure 7.2. The *P. poae* genome sequence would enable us to identify transcriptional factors and other novel regulons which are associated with insect virulence and other secondary metabolite gene expression. The production of secondary metabolites (pyoverdine and phenazines) can be improved by manipulating their transcriptional factor activators/repressors. For example, the role of quorum sensing (QS) in regulating the production of secondary metabolites in Pseudomonads could be explored (Laue *et al.,* 2000). Similarly, identification of signal molecules and characterization of quorum sensing targets in the *P. poae* genome would help to determine if QS plays a role in insect pathogenesis and other biocontrol activities. In Pseudomonads, many of the secondary metabolite biosynthetic genes are organised as clusters, which could facilitate the construction of strains with the potential to synthesise a range of antagonists, as reviewed by Haas *et al* (Hammer *et al.,* 1997; Mavrodi *et al.*, 1998; Bangera & Thomashow, 1999; Thompson *et al.,* 1999; Haas *et al.,* 2000). Hence, introducing new secondary metabolite genes into *P. poae* should be considered in the future. Both transcriptional and post-transcriptional regulation of these genes must be reorganised to achieve optimal production of secondary metabolites. Overexpression of *P. poae* toxins (such as Tc, RhsA2 and AprX) in *E. coli* and their expressed protein product might also be utilised directly as bio-pesticides. Furthermore, transgenic plants could be constructed to create recombinant proteins to protect the plant from attack of pests and pathogens.

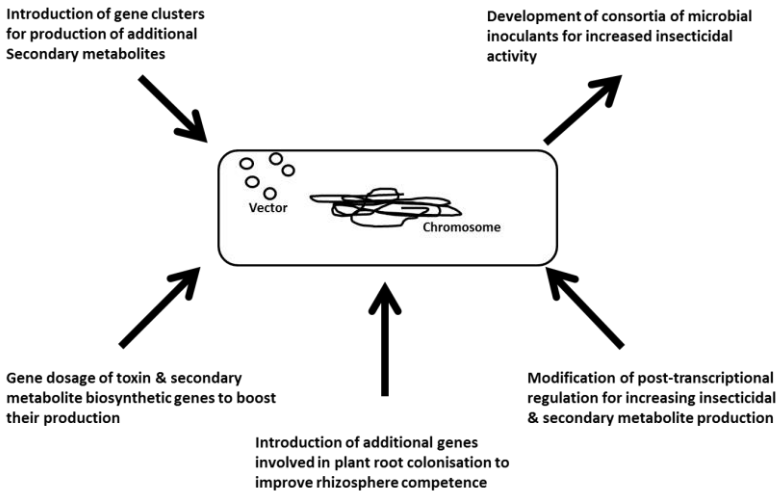


Figure 7.2: Schematic diagram depicting the strategies being followed to develop improved biocontrol strains of *Pseudomonas*.
Figure designed and published by Ultan F Walsh (Walsh *et al.,* 2001).

The *P. poae* genome revealed the presence of determinants that may elicit induced systemic resistance in plants including iron chelators (pyoverdines), antimicrobials (phenazines) and lipopeptides (Djavaheri *et al.,* 2009; Verhagen *et al.,* 2010). Similar to other rhizobacteria, *P. poae* likely evolved the ability to compete in the rhizosphere through use of a range of systems including chemotaxis, motility and high affinity iron chelators (e.g. siderophores such as pyoverdines) (Table 6.6, 6.8 & 6.9). This would enable us to further explore the use of the *P. poae* strain as seed coatings for inoculum releases in the rhizosphere. However, in this study, the failure of a seed soak method during delivery of *P. poae* on sugar beet seeds suggests that a future aim should be to improve the seed coating with appropriate pelleting (either use of alginate beads, peat carrier and seed priming). Further investigation is required to confirm bacterial viability on dried seeds and whether an improved inoculation method allows bacterial cells to survive for long periods under storage, at concentrations high enough to afford biocontrol. Along with effective formulation, monitoring of *P. poae* colonisation and their competence in the rhizosphere, using green fluorescent protein (GFP) and/or bioluminescence techniques, would be beneficial (Normander *et al.,* 1999; Bloemberg *et al.,* 2007).

Besides these, induced systemic resistance (ISR) elicitors of many bacterial strains (including *Bacillus*, *Pseudomonas* and others) are well known to modulate plant hormone signalling pathways to alter plant defence directly and indirectly. They directly activate JA and ET mediated plant defences which induce expression of defence related genes and also increase green leaf volatiles (Pineda *et al.,* 2010). Depending on abiotic and biotic factors, these plant mediated responses have positive, negative and neutral effects on the herbivore performance. While in the

case of indirect response, ISR-primed plants also attract natural predators which affect herbivore colonisation.

Olfactometer assays in this study reported the modified behaviour of aphids, which suggested colonisation of *P. poae* on the plant leaves generates strongly repellent sites through the production of deterrent semiochemicals (Figure 4.8). These results suggested that *P. poae* ISR elicitors can interfere with JA and ET signalling pathways and also modulate green leaf volatile production. One of the chemical defences mediated by the JA hormone stimulates production of repellent, anti-nutritive, or toxic compounds which prevent further plant infestation (Howe & Jander, 2008). The plant hormones jasmonic acid, ethylene, and salicylic acid are the main phytohormones regulating defence against different attackers, including herbivorous insects such as caterpillars and aphids (Pieterse & Dicke, 2007). Further studies on *P. poae* colonisation in the plant rhizosphere will help to understand mechanism of plant systemic resistance, which in turn can affect aphid performance above ground.

Genomic and mutagenesis approaches targeting key genes involved in the interaction between plants and associated beneficial bacteria systems might also enhance *P. poae* rhizosphere competence and persistence in various ecological niches. In summary, the current knowledge will help with the development of *P. poae*-based plant products, which can provide long term control of insects and phytopathogens simultaneously in an IPM framework.

7.5 Resistance management strategies

Resistance management in *M. persicae* is difficult due to unpredictable changes in the severity of aphid attack from year to year, continuous changes in the incidence of particular resistance mechanisms, and the ability of this aphid to utilise a large number of plant species as hosts. It is also essential to anticipate how attacks by different pests, other aphid species and caterpillars, may affect control strategies for *M. persicae* (Insecticide Resistance Action Group, 2003).

Although not used against aphids, resistance development against microbial pathogens has been most commonly reported in the case of *B. thuringiensis*. At least 16 insect species were found to be resistant to *B. thuringiensis* endotoxins under laboratory conditions and field-evolved resistance has been documented in noctuids such as *Spodoptera frugiperda*, *Busseola fusca* and *Helicoverpa zea* (Tabashnik *et al.,* 2014). Furthermore transgenic plants which express microbial genes were subsequently introduced and *B. thuringiensis* cotton and *B. thuringiensis* maize is now available in 13 and 9 countries, respectively, grown on 42.1 million ha of land (Shelton *et al.,* 2008). However, field-evolved resistance to *B. thuringiensis* crops is rapidly growing as a result of

an increase in the frequency of resistance alleles (Tabashnik *et al.,* 2008). Hence, two effective measures have been used to control field resistance in *B. thuringiensis*: 1. using high bacterial dose/refuge insect and 2. gene pyramiding in transgenic plants (expression of more than one toxin) (Zhao *et al.,* 2003;Manyangarirwa, *et al.,* 2006). Later, it has been shown that gene pyramiding may not be a sustainable strategy (Tabashnik *et al.,* 2014); therefore, other biocontrol strategies such as the use of predators and parasitoids and crop rotation strategies should be incorporated in the management plan. Another study described resistance in the moth *Cydia pomnella* against GV baculovirus in the field (found in Europe) due to overuse of the product which blocked virus replication by impairment of brush border cell receptors (Asser-Kaiser *et al.,* 2010). However, there have been no reports of field resistance development to entomopathogenic fungi or nematodes. Nevertheless, occurrence of natural resistance mechanisms in insects against fungi and nematodes suggests that resistance to these pathogens cannot be overlooked (Wilson, *et al.,* 2001; Wilson *et al.,* 2002; Kunkel *et al.,* 2004).

Hence, Combinations or rotations of all cultural, chemical and biological control methods have been utilised in aphid IPM strategies to combat resistance. The use of cultural control methods such as slow-release fertilizers, protective covers for seedlings and silver-coloured reflective mulches with correct timings for release of predators and parasitic wasps resulted in effective aphid control (Bugg *et al.,* 2008). Moreover, the combined use of insecticidal soaps and petroleum-based horticultural oils or plant-derived oils such as neem or canola oil are commonly utilised to control infestation of woolly apple aphid, green apple aphid, rosy apple aphid, mealy plum aphid, and black cherry aphid (Flint, 2013).

P. poae as a biocontrol agent as described in this PhD could be utilised in IPM strategies. It is likely that resistance most often develops to pesticides/biocontrol agents that are initially very effective and frequently used. Hence, pesticide resistance management aims to slow or prevent the development of resistance. Resistance development in pest populations is influenced by biological, ecological, genetic, and operational factors. In laboratory conditions, resistance can be evaluated by comparing differences in susceptibility between a susceptible population and populations which show some degree of resistance on application of pesticide/biocontrol agent. Likewise, baseline susceptibilities to *P. poae* (obtained from dose-response assay) could identify probable resistance problems before they take hold, and enable us to propose pesticide-use strategies to anticipate resistance development.

Therefore, it is worthwhile evaluating resistance development in aphid populations by application of *P. poae* under both laboratory and field conditions.

Several resistance management (based on pesticide strategies, (Hoy, 2008))approaches have been described which could be utilised as future work from this study:

1. Management by moderation

Moderation is a form of limiting the use of a pesticide or persistence. In general, moderation is employed in concert with IPM practices such as using treatment thresholds, using lower dosages of pesticides (when appropriate), spraying only specific pest generations or growth stages, treating only limited areas, maintaining unsprayed areas as refuges for susceptible individuals and using pesticides with shorter residual or lower toxicity to important beneficial populations.

Likewise, the optimal *P. poae* dose (at moderate level) would be applied on aphids present at damaging levels where they could easily target the specific stage of aphid life cycle such as nymphs or three instars to reduce chances of resistance. The application of the optimal bacterial dose only on infected crops would maintain selection pressure at low levels and prevent resistance development.

2. Management by multiple attack :

This approach involves using either mixtures or rotations of pesticides to prevent resistance. In general, mixtures of insecticides have resulted in pest populations developing high frequencies of resistance to all pesticides in the mixture. Crop rotation is difficult to implement due to the wide host range of green peach aphid. Therefore, it is desirable to combine cultural, mechanical, biological, and chemical control measures into a practical pest control program.

I have discussed possible application methods of *P. poae* as a foliar spray, pouring or drenching soil, and seed coatings, all which could be explored in the context of integrated pest management. To combat resistance development, different modes of *P. poae* application with combinations of cultural and chemical methods, within an IPM framework, would be beneficial. A rotation technique could be employed to use different transgenic plants encoding one or more *P. poae* toxins with another biocontrol approach such as use of predators and parasitoids in every season. Such variation in mode of action of biopesticides would prevent the build-up of resistance in aphids against specific biocontrol agent. Future research should investigate the interaction of *P. poae* with natural predators and other IPM strategies which could show a synergistic effect on the suppression of aphids.

7.6 Conclusions & Future work

The data generated from this study, coupled with previously unpublished work has identified the plant derived aphid- killer *Pseudomonas poae* and described several different categories of genes that could be investigated in the future to further understand the killing mechanism. Many of the bacterial genes that were differentially expressed during pathogenesis were involved in iron acquisition, virulence (toxins & other determinants), oxidative stress, motility and nutrient assimilation and utilisation. The bacterial growth data from infected aphids, differential expression of aphid saliva and gut specific genes suggested that the gut constitutes a good environment for the development of this *P. poae* bacterial strain inside aphids. This forms the basis of future work to investigate the localization of *P. poae* inside aphid gut, for example by using a dual-label *in situ* hybridization technique employing specific 16S ribosomal RNA probes for the gut symbiont *Buchnera* as well as *P. poae*. Also, it can be envisaged that *P. poae* would proliferate in other parts of aphid body and this could be tested by using a microinjection technique. The direct injection of *P. poae* into the hemolymph would be appropriate to determine 1) whether *P. poae* is able to survive in the hemolymph and avoid host immunity, 2) whether *P. poae* can invade internal tissues of the infected host and be localised in bacteriocytes or sheath cells.

Nevertheless, the weak innate immune response of aphids could be the consequence of an evolutionary cooperation to avoid elimination of the symbiotic partners'. In terms of immune response, I hypothesize that invasive bacteria have to deal with the host lysosomal system during colonization and other functional detoxification genes on which more attention should be paid in the future. Further investigation on genomic and physiological analysis of symbionts during pathogenesis might provide insights on nutritional interactions in insect-microbial symbiosis.

The RNA profiling of *P. poae* found that a large proportion of the hypothetical genes were differentially expressed. Many of the hypothetical genes were found to be in close proximity to known toxins, membrane transporter and metabolic enzymes, suggesting a proposed role in regulation. In the future, knockout mutants of selected candidate genes could be tested for their ability to infect and colonize aphids. Further analysis may elucidate their function and work out if they are real proteins that are expressed. Potentially, these genes could be the most interesting as a role has yet to be characterised for them. Moreover, the identification of putative secondary metabolite synthesis genes with the antiSMASH tool and other candidate genomic islands by IslandViewer 4 tool would enables us to identify other novel genetic elements in insect pathogenesis. Similarly in the aphid transcriptomic data, a limited number of genes that categorized as uncharacterised proteins were differentially expressed. Gene annotation of a few

uncharacterised proteins described the molecular function and cell component features which could be utilised in future for their role in the bacteria –insect interaction.

The current work addressed biocontrol capabilities of *P. poae* and their potential to modulate plant defences through a variety of mechanisms. Many studies showed the classical defence hormones, SA, JA, and ET, and various other growth-related hormones (CK, auxin, and brassinosteroids), are also involved in fine-tuning these microbe–plant–insect interaction (Erb *et al.,* 2012; Meldau *et al.,* 2012; Giron *et al.,* 2013). In future, the identification of bacteria (*P. poae*) and insect (aphid) determinant factors together with genetic, genomic and biochemical tools could be used in the study of phloem-feeding induced defence response and plant signalling pathways associated with both aphid attack and bacterial infection. Moreover, natural ecological settings will help to elucidate the underlying mechanisms of microbe–plant–insect interactions.

The comprehensive dose-response data of different resistant clones revealed no strong correlation between multiple resistance mechanism and bacteria susceptibility patterns. However, only *P. poae* bacteria showed some consistent toxicity towards resistant clones therefore more research is certainly needed to determine the cost/benefit balance of the resistance mechanisms in aphids after the exposure of *P. poae* bacteria. Future *in planta* assays would be conducted within the range of higher and lower doses of susceptible aphid clones to characterise the standard dose of *P. poae.* Selection of a moderate dose needed to control aphid infestation at higher efficacy rate will avoid chances of resistance (reduce selection pressure).
The present work demonstrates that *P. poae* has a potential role to play in development of future integrated pest management strategies. The schematic presentation of events from isolation of potential *Pseudomona*s biocontrol strains through their development and improvement to their marketing as plant protection products (PPPs) is shown in Figure 7.3.

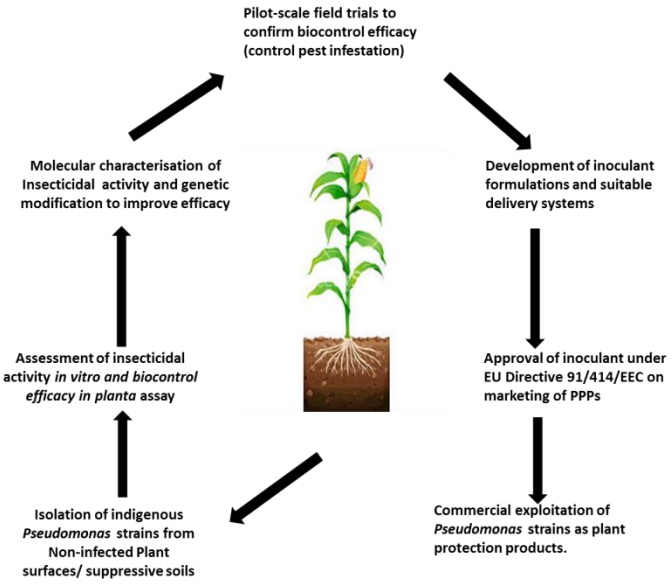


Figure 7.3: Sequence of events from the isolation of potential *Pseudomonas* biocontrol agents to commercial exploitation. Figure designed and published by Ultan F Walsh (Walsh *et al.,* 2001).

The efficacy of these bacteria is usually related to an effective mode of application in the field. This has led to a special formulation of bacteria-based bio insecticides, with the aim of maximizing shelf-life, improving dispersion and adhesion, reducing spray drift and above all enhancing efficacy.

A range of adjuvants have been available for microbial formulations such as phagostimulants, carriers, pH buffers, dispersants, antifoam agents and attractants. (Brar *et al.,* 2006). Additionally, carbon sources and minerals play an important role in secondary metabolite production by *Pseudomonas* **b**io**c**ontrol **a**gents (BCAs), supporting the notion that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy (Duffy & Défago, 1999). The edaphic parameters play an important role in targeting bacterial inoculants in rhizosphere in order to support biocontrol efficacy. The soil amendments or substrates with minerals such as zinc or priming inoculants with media amendments during fermentation, improved biocontrol efficacy of *Pseudomonas fluorescens* (Duffy & Défago, 1997*;* Duffy & Defago, 2000*;* Ownley *et al.*, 2003). Therefore it is essential that further research is conducted on the development and optimisation of *P. poae* inoculant formulations, which will be compatible with current seed coating technologies.

In addition to optimizing efficacy, *P. poae* must be assessed for their effect on human health, the environment and non-target organisms. Many studies stated that genetically modified *Pseudomonas* BCAs do not hamper indigenous bacterial consortia (usually beneficial and symbiotic species) (Barea *et al.,* 1998; Edwards *et al.,* 1998; Mar Vázquez *et al.,* 2000). However, another research study revealed *Pseudomonas* BCAs can affect the growth and subsequent

nodule occupancy of certain *Sinorhizobium meliloti* strains in gnotobiotic systems (Niemann *et al.,* 1997).

Further investigation should be carried out to investigate the interaction of *P. poae* bacteria with the natural microbiota of soil and the insect gut. This would provide rapid assessment of *P. poae* killing efficacy of insects under field conditions. Such an approach may avoid inconsistency of *Pseudomonas*-based products in the field from the beginning. The preservation of the environment and human health and the need to manage the development of insect resistance to pesticides are additional concerns. As a result, the incorporation of bio-based insecticides in combination or in rotation with synthetic formulations is strongly recommended. At the end, marketing of *P. poae* based products as eco-friendly alternatives to chemicals will depend on the generation of biosafety data essential for the registration of biocontrol agents (European Union directive 91/414/EEC).

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