# PLANT-BENEFICIAL FLUORESCENT PSEUDOMONADS WITH INSECTICIDAL ACTIVITY: MOLECULAR TRAITS AND ECOLOGY OF INSECT-ASSOCIATED LIFESTYLES

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″Any sufficiently advanced technology is indistinguishable from magic″

″Now, I’m a scientific expert; that means I know nothing about absolutely everything″

- *Sir Arthur C. Clarke*

″Estamos muertos desde el mismo momento en que dejamos de soñar″

- *Gritando en Silencio*

Dedicated to my parents and my grandma, Celia.

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# SUMMARY

This thesis focuses on a special group of plant-beneficial pseudomonads with insecticidal activity. Bacteria belonging to the *Pseudomonas fluorescens* group are excellent root-colonizers with many plant beneficial effects. These bacteria can promote plant growth through the production of hormones and increase the availability of certain soil nutrients. Fluorescent *Pseudomonas* are also able to control the emergence of soil-borne diseases mainly by the production of antimicrobial secondary metabolites and to induce systemic resistance in the plant host rendering it ready for the attack of pathogens or predators. Intriguingly, two species within the fluorescent pseudomonads, *P. chlororaphis* and *P. protegens*, possess the ability to colonize and kill Lepidopteran insects in addition to all these plant beneficial activities. In the last decade, several factors contributing to the insecticidal activity of these fascinating bacteria were identified, and insecticidal pseudomonads have emerged as promising candidates for the biological control of soil-derived insect pests for which no satisfactory control methods exist.

The major aims of this thesis were to study the nature of *Pseudomonas*-insect interactions and its ecological significance, and to investigate, in a susceptible insect species, the pathogenicity process and the factors required at different phases during insect colonization and killing.

In the first part of the thesis, we investigated whether the inability, of model strain *P. protegens* CHA0, to kill certain insect species, is due to its failure to persist in the animals. Based on feeding assays in combination with bacterial monitoring we showed that *P. protegens* CHA0 is highly lethal to larvae of *Plutella xylostella* (Lepidoptera) and *Pieris brassicae* (Lepidoptera) while being pathogenic, but less effective against larvae of *Delia radicum* (Diptera). *P. protegens* CHA0 had no effect on larvae of *Otiorhynchus sulcatus* (Coleoptera). However, *P. protegens* CHA0 persisted from larval to adult stage in all these

species and was transmitted to a new plant host by *D. radicum* flies. These findings indicate that insecticidal pseudomonads can establish different relationships with insects ranging from exploiting insects as food source to using them as vectors for dispersal.

A central part of the thesis concentrated on identifying the specific sets of genes a pseudomonad needs when colonizing a plant or an insect hosts, and to identify the specific role of individual factors during insect colonization and throughout disease progression. To this end, a large-scale transcriptomics dataset of *P. protegens* strain CHA0 was generated which includes data from the colonization of wheat roots, the gut of *P. xylostella* after oral uptake and the *Galleria mellonella* hemolymph after injection. The transcriptomic profiles strongly varied depending on the environment. Furthermore, we could associate specific factors to different hosts or different stages of insect infection. In addition, new traits contributing to insecticidal activity were identified, such as effector proteins (toxins) released by two-partner secretion systems (TPS). Their role during infection of *P. xylostella* was verified using a mutational approach. Altogether, this data allowed us to propose a comprehensive insect colonization and pathogenesis model for *P. protegens* CHA0.

The ecological relevance of non-pathogenic interactions with insects described in the first part of the thesis, and the natural association of *P. protegens* and *P. chlororaphis* to insects, still remains unknown. To address these questions, we searched for insecticidal *Pseudomonas* in arthropods collected from a wheat field, a potato field and neighbouring grassland. We found that *P. protegens* and *P. chlororaphis* are naturally present in healthy insects and myriapods and isolated a set of new strains from arthropods, soil and roots. Although all strains, independently of their host of origin or their phylogenetic position, had oral activity against *P. xylostella*, the insecticidal capacities of different *P. chlororaphis* isolates were not as homogeneous as observed for *P. protegens*.

Phylogenetically closely related *P. chlororaphis* strains differed in insect killing speed and efficiency. We hypothesize that the lower insecticidal activity observed for two Coleoptera isolates could be related to mutations in key insecticidal factors, such as the Fit toxin and TPSA proteins, that we discovered using a single nucleotide polymorphism analysis based on the whole genomes. These findings point towards an order-specificity or adaptation to certain insect hosts and show the ubiquitous nature of these special pseudomonads.

This thesis substantially improves our knowledge about the pathogenesis of insect infecting *Pseudomonas* and the ecology of arthropod-*Pseudomonas* relationships. The novel information we gained is of great scientific, but also agricultural and environmental value, since it is highly important for the development of new biocontrol tools within the frame of a sustainable agriculture relying on environmentally friendly pest control methods.

# ZUSAMENFASSUNG

Im Rahmen dieser Dissertation wurden Pseudomonaden mit insektizider Aktivität untersucht, eine spezielle Gruppe innerhalb der fluoreszierenden Pseudomonaden. Diese Bakterien sind bekannt sind für ihre Fähigkeit Wurzeln zu kolonisieren und auch für ihre vielen nützlichen Effekte auf Pflanzen. So können sie das Pflanzenwachstum fördern und die Verfügbarkeit von gewissen Nährstoffen im Boden erhöhen. Fluoreszierende Pseudomonaden sind auch fähig Pflanzen vor bodenbürtigen Krankheiten zu schützen und in ihnen systemische Resistenz gegen Pathogene und Insekten zu induzieren. Faszinierenderweise gibt es zwei Arten unter ihnen, *Pseudomonas protegens* und *Pseudomonas chlororaphis*, die nicht nur alle diese nützlichen Eigenschaften haben, sondern zusätzlich noch die Fähigkeit besitzen, Lepidopterenlarven zu besiedeln und zu töten. Im letzten Jahrzehnt wurden mehrere insektenpathogene Faktoren bei diesen Bakterien entdeckt und insektizide Pseudomonaden werden als vielversprechende Kandidaten für die Entwicklung von Biokontrollprodukten angesehen, welche gegen schwer zu bekämpfende bodenbürtige Insekten eingesetzt werden können.

Die Hautpziele dieser Arbeit waren, erstens die Natur der *Pseudomonas*-Insekten Interaktion und ihre ökologische Bedeutung zu untersuchen und zweitens, in einer anfälligen Insektenart, den Prozess der Pathogenese zu studieren mit dem Ziel die Zeitpunkte, an denen verschiedene Faktoren wirken zu identifizieren.

Im ersten Teil der Dissertation haben wir untersucht, ob die Unfähigkeit des Modell-Stammes *P. protegens* CHA0 gewisse Insektenarten zu töten daran liegen könnte, dass die Bakterien im Innern des Insektes nicht überleben. Aufgrund von Fütterungsversuchen mit anschliessendem Monitoring der Bakterien im Insekt konnten wir zeigen, dass die beiden Lepidoptera Arten *Plutella xylostella* und *Pieris brassicae* hoch anfällig auf CHA0 sind, die Diptera Art *Delia radicum* weit weniger anfällig und die Colepotera Art

*Otiorhynchus sulcatus* weitgehend resistent ist. Interessanterweise konnte das Bakterium aber in allen vier Arten vom Larven- über das Puppenstadium bis zum adulten Insekt überleben und wir konnten zeigen, dass CHA0 sogar von adulten *Delia* Fliegen auf neue Wirtspflanzen übertragen werden kann. Unsere Resultate deuten darauf hin, dass insektizide Pseudomonaden verschiedene Arten von Beziehungen mit Insekten eingehen können und diese in manchen Fällen als Nahrungsquelle, in andern als Vektoren für ihre Verbreitung nutzen können.

Ein zentraler Teil dieser Arbeit beschäftigte sich damit zu untersuchen, welche spezifischen Gene die Pseudomonaden für die Besiedelung von Wurzeln und welche sie für die Kolonisierung von Insekten brauchen. Dazu sollte herausgefunden werden, welche Faktoren die Bakterien zu welchem Zeitpunkt der Kolonisierung und des Infektionsprozesses brauchen. Zu diesem Zweck haben wir ein einen Vergleich von *P. protegens* CHA0 Transkriptomen gemacht d.h. von Bakterien während der Besiedelung von Weizenwurzeln, während verschiedener Phasen der Besiedelung des Darms von *P. xylostella* nach oraler Applikation sowie von Bakterien nach Injektion in die Hämolymphe von *Galleria mellonella*. Die Transkriptome unterschieden sich stark voneinander in Abhängigkeit des Wirtes und der verschiedenen Infektionsphasen und wir konnten einzelne Pathogenitätsfaktoren bestimmten Phasen der Infektion zuordnen. Zudem gelang es uns, neue Faktoren zu identifizieren wie z.B. Effektoren (Toxine), die von Zwei-Partner-Sekretionssystemen (TPS) gebildet werden und ihre Rolle in der Pathogenese mittels Deletionsmutanten zu verifizieren. Die Erkenntnisse dieses Dissertationsteiles ermöglichten uns, ein umfassendes Modell für die Insektenkolonisierung und -pathogenität von *P. protegens* CHA0 aufzustellen.

Die ökologische Relevanz von nicht-pathogenen Interaktionen mit Insekten, wie sie im ersten Teil der Dissertation beschrieben wurden, sowie die Frage, ob *P. protegens* und *P.*

*chlororaphis* in der Natur mit Insekten assoziiert sind, sind noch ungeklärt. Diesen Fragen wurde im letzten Teil der Dissertation nachgegangen. Dazu untersuchten wir verschiedene Arthropoden, die wir in einem Weizenfeld, einem Kartoffelfeld und einer benachbarten natürlichen Wiese gesammelt hatten. Tatsächlich konnten wir beide *Pseudomonas*-Arten von gesunden Tieren, die zu verschiedenen Insekten- und Myriopoda-Arten gehören isolieren. Wir verglichen die phenotyptischen Eigenschaften einer Sammlung von Pflanzen- und Insekten-Isolaten. Wir stellten fest, dass unabhängig vom Wirt, von dem sie isoliert worden waren und von ihrer Position im phylogenetischen Stammbaum, alle Isolate orale Aktivität gegen Larven von *P. xylostella* haben, aber dass die *P. chlororaphis* Isolate viel diverser bzgl. insektizider Aktivität sind als wir es für *P. protegens* beobachten konnten. Phylogenetisch sehr nahe verwandte *P. chlororaphis* Isolate unterschieden sich deutlich in ihrer Effizienz und Geschwindigkeit *Plutella* Larven zu töten. Wir vermuten, dass die reduzierte insektizide Wirkung, die wir bei zwei Käferisolaten festgestellt haben, auf einige Mutationen in für die Pathogenese wichtigen Genen zurückzuführen sein könnte. Eine „single nucleotide polymorphism“ Analyse der Genome zeigte, dass die Käferisolate, im Vergleich zu hoch aggressiven Wurzelisolaten, aminosäureverändernde Mutationen des Fit Toxingens sowie zweier TPS Gene aufweisen. Diese Resultate deuten auf eine mögliche Spezifizität für verschiedene Insektenordnungen, respektive auf eine Adaptation an verschiedene Insektenwirte innerhalb dieser speziellen Gruppe von Pseudomonaden hin und verdeutlichen ihre unglaublichen Fähigkeiten, sich an verschiedene Umgebungen anzupassen.

Diese Dissertation verbessert unser Wissen über die Pathogenese von Insekten-infizierenden Pseudomonaden und die Ökologie von *Pseudomonas*-Arthropoden Beziehungen wesentlich. Die gewonnenen Informationen sind nicht nur von Bedeutung für die Wissenschaft, sondern auch für die Landwirtschaft und die Umwelt, da sie äusserst

wichtig sind für die Entwicklung von *Pseudomonas*-Bakterien als neue Biokontrollwerkzeuge im Rahmen einer nachhaltigen Landwirtschaft und der Nutzung von umweltfreundlichen Methoden für die Kontrolle von Schadinsekten.

# Chapter 1

General Introduction

# 1. Current and future challenges in agriculture

In the year 2030, there will be ~8.5 million people on the planet [1] who will need to be fed. About 50% of the food supply worldwide comes from wheat, soy, rice, potato and maize crops [2–4] but in order to feed the rising population, a 2.4% yield increase per year is needed. However, almost no country is able to fulfil this requirement partially because of high yield losses [3]. Global yield losses are due to unfavourable weather conditions, climate change, pests, pathogens and poor harvesting and storage practices [2, 5–7]. Plant pathogens and pests include viruses, viroids, oomycetes, bacteria, protists, fungi, nematodes, molluscs, insects, vertebrates and parasitic plants. It is estimated that they cause between 17 and 30% of annual yield losses in wheat, rice, potato, maize and soy worldwide [4, 8].

The increase of crop production by freeing new land is not environmentally friendly, therefore, yield must increase and losses have to be averted [5, 9]. Several control methods are used for preventing pests such as sanitation, solarisation, resistant crop cultivars, crop rotation, biofumigants, soil amendments, management of suppressive soils, active managing of the soil nutrients and plant microbiota, or genetically modified plants [9]. However, pathogens, weeds and pest insects are usually controlled using pesticides due to their high effectivity. Pesticides are costly to produce, not durable and a hazard for the environment as they are applied in high concentrations and reach water sources damaging other ecosystems and the resident wildlife. In the past few years many pesticides have been banned or are under investigation for potential effects on non-target organisms and harm to the environment. They can also be dangerous for human health because, often, farmers in developing countries do not have access to the safest chemicals or appropriate equipment [10]. Additionally, pests are increasingly becoming resistant to pesticides which makes the need for better pest management practices evident [9–12]. More recently,

the use of organisms that are antagonists or pathogens of major pests, known as biological control agents, has become more frequent [9, 11]. Integrated pest management aims to use all the physical, cultural and biological measurements available, before using chemical pesticides, in order to build a more sustainable agriculture in the context of a growing population.

## 2. The “soil-ution": soil microbes as an answer to the food crisis.

Suppressive soils are naturally resistant to the development of certain diseases, even if the pathogens are present in the environment. It has been shown that diseases produced by oomycetes (*Pythium* spp.), bacteria (*Ralstonia solanacearum*, *Streptomyces scabies*), fungi (*Rhizoctonia solani, Gaeumannomyces graminis*, *Fusarium oxysporum*, *Thielaviopsis basicola* and *Phytophtora cinnamoni*), or nematodes (*Meloidogyne incognita*) are averted in such suppressive soils. Disease suppression is mostly due to specific soil compositions and conditions, such as the pH and/or the resident microbial communities of the soil that can produce antibiotics, promote plant growth or induce systemic resistance in the plant hosts. Suppressive soils were the first environment where plant-microbe beneficial interactions were studied for disease prevention. For example, the presence of *Pseudomonas* spp. and non-pathogenic *F. oxysporum* is very important to supress Fusarium wilt caused by *F. oxysporum*. Another common disease is the wheat take-all decline caused by *G. graminis* var. *tritici* which is sensitive to the presence of *G. graminis* var. *graminis*, amoeba, actinomycetes, *Trichoderma* spp., *Bacillus* spp. and *Pseudomonas* spp. [13, 14]. Even though beneficial microbes are more abundant in suppressive soils, they are also present in non-suppressive soils, but in lower numbers [14–17].

The rhizosphere is the soil surrounding the root which is directly influenced by the

plant [18]. Plants are able to change conditions in the rhizosphere soil through the secretion of a variety of organic compounds, which also determine the kind of microbes they recruit from the soil into the rhizoplane. This mutualistic interaction is beneficial for both plant-host and microbes, because the plant provides a carbohydrate source with the exudates and the microbes enhance the host fitness [19]. Microbial agents can promote plant growth (section 2.1), induce systemic resistance in the plant (section 2.2.2), and control soil borne diseases (section 2.2) and pest insects (section 2.3). Therefore, the soil microbes could be part of the answer to solve the current most prominent problems in agriculture and food production (i.e. yield increase and loss reduction). The rhizosphere is 10 to 1000 times richer in microbes than bulk soil with a high diversity within the Firmicutes and Proteobacteria phyla [19, 20]. The rhizosphere and also bulk soil are rich in beneficial fungal and bacterial species and other organisms that enhance plant fitness.

This thesis focusses on plant-beneficial pseudomonads with special emphasis on *Pseudomonas* species displaying plant growth promoting, disease suppressive and additionally also insect pathogenic properties (Fig. 1). *Pseudomonas* are very versatile bacteria with a wide range of metabolic and catabolic activities, which enables them to colonize very different environments and establish pathogenic and beneficial interactions with animal and plant hosts [21]. Specifically, members of the fluorescent *Pseudomonas* group are well-known for their manifold beneficial activities. They have been associated with natural disease suppressiveness of certain soils and have been used for decades as biofertilizers and biocontrol agents (see the following chapters).

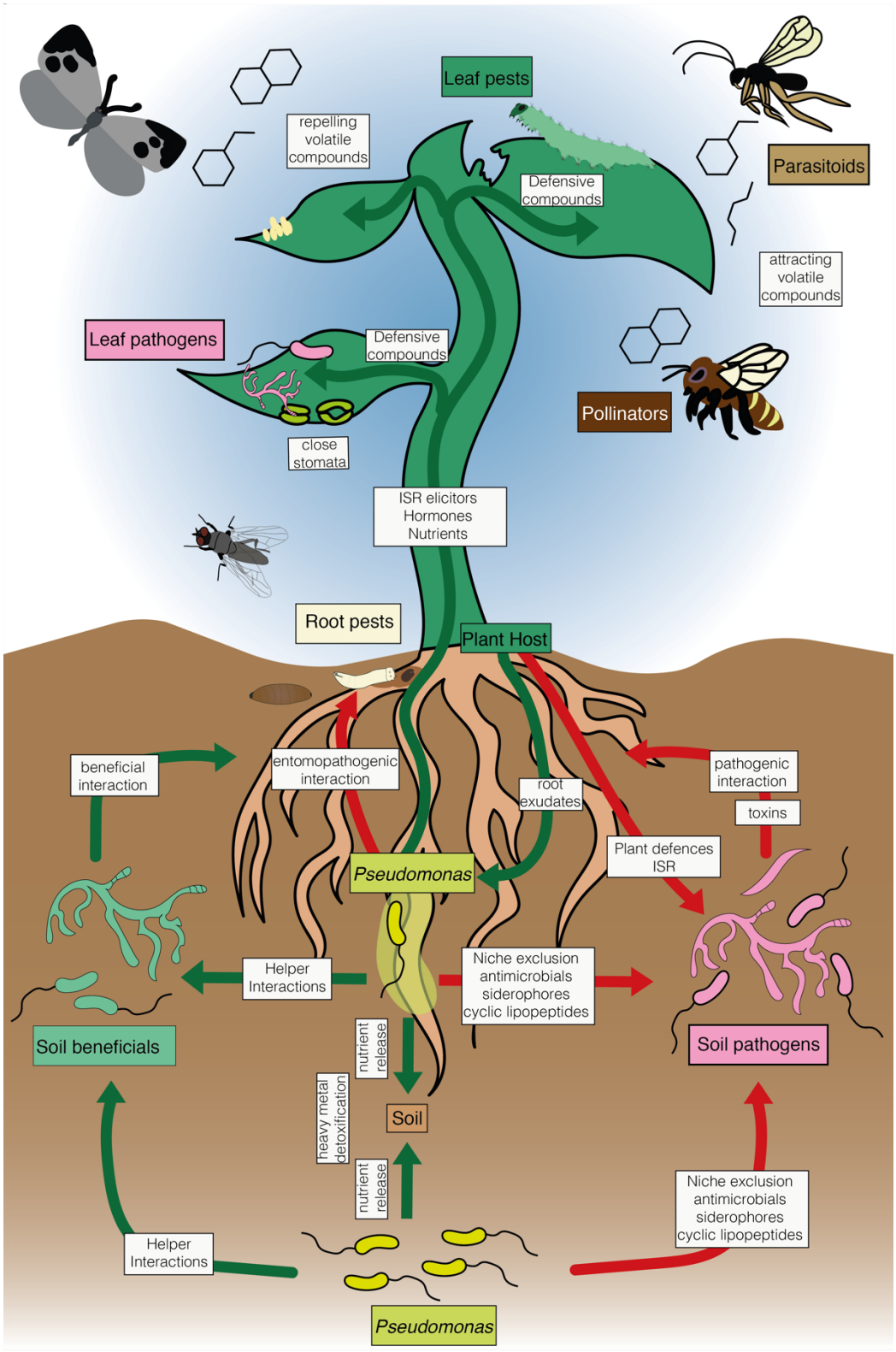


**Figure 1. Manifold beneficial impacts of *Pseudomonas* on plant hosts.** *Pseudomonas* species can either live freely in the soil or associate to plant roots forming microcolonies which use root exudates as nutrient source. They can promote plant growth directly by the production of growth hormones or indirectly by making soil nutrients available, helping beneficial microbes to establish an interaction

with the plant or by controlling pathogens and pest insects. They can control soil-borne pathogens through competitive colonization or production of antimicrobials and siderophores. *Pseudomonas* can also produce toxins that kill root pests. They can further induce systemic resistance in the plant resulting in an activation and acceleration of its fight against pathogens e.g. by producing effector molecules, closing stomata or accumulating toxic defensive compounds in damaged areas. *Pseudomonas* can also modify volatile production of the plants to repel pest insects or to attract pollinators and hyperparasites of pest insects. Green arrows indicate positive interactions; red arrows antagonistic interactions and the white boxes the *Pseudomonas* effect in a particular interaction.

## 2.1. Plant Growth Promoting Rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are natural inhabitants of the rhizosphere that can enhance plant growth and increase soil quality. Microbes can improve soil quality by heavy metal detoxification, i.e. rhizoremediation, that has been studied in *Bacillus* species and certain genetically engineered *Pseudomonas* for land improvement [22, 23]. Also, PGPR can increase salt tolerance of the plant by hijacking ethylene production which is the hormone responsible for senescence under stress conditions. For example, mung beans growing in fields with high salinity and treated with *Pseudomonas* spp. and *Rhizobium* spp. showed improved fitness compared to untreated plants [22, 24]. Additionally, PGPR such as certain *Rhizobium*, *Azospirillum*, *Bacillus*, *Agrobacterium* and *Pseudomonas* species can increase the solubility of some nutrients and affect the uptake systems of the plant. For example, PGPR can increase phosphorus solubility by lowering the pH through the production of organic acids and increase iron availability by the production of siderophores (further discussed in section 2.2). Some bacteria belonging to the *Rhizobium* species can even fix atmospheric nitrogen and render it available for the plant, thereby increasing its fitness [19, 22].

PGPR can also directly contribute to plant growth through the production of hormones such as auxin, cytokinin and ethylene, volatiles and growth regulators [19, 20].

The way that bacteria influence the plant is species dependent and can have different consequences for the plant. For example, *P. fluorescens* WCS365 auxins boost radish root growth [25] and *P. fluorescens* G20-18 cytokinins promote growth of wheat and radish plants [26]. They can influence the root architecture and structure of the cells through the production or regulation of phytohormones leading to an increase in root surface and an improvement in nutrient uptake. In *Fabaceae* the root tip is where the rhizobia start colonization while in *Poaceae*, PGPR obtain the nutrients from the lateral roots. Therefore, different bacteria will tend to produce hormones that favour their preferred habitat within the plant root. The plant-bacteria interactions are, in some cases, very specific and both partners can mutually regulate their transcriptome or metabolome through the production of exudates or volatiles [19].

Other bacteria do not influence the plant growth promotion directly, but they can favour beneficial organisms to establish their interactions [20]. This is the case of *P. fluorescens* BBc6R8 as helper of mycorrhizal fungi [27] or *P. trivialis* 3Re27 as *Rhizobium* helper bacterium [28].

## 2.2. Disease suppression by bacterial biocontrol agents and its exploitation in agriculture

Crop losses by pathogens and pests are a major challenge in the development of sustainable agriculture. Therefore, a boost in crop growth by PGPR is not enough to provide for the food requirements of the rising population. Moreover, pest insects can be attracted to PGPR treated plants due to an increase in nutrient availability. Fortunately, in addition to their plant growth promotion activity, some PGPR can also control diseases. Biological control agents (BCA) are becoming more and more popular as an alternative to, or complementation of, chemical pesticides, because they are more environmentally

friendly, in most cases more specific than chemicals, and cause no harm to non-target organisms or human health [29, 30]. Biological control agents can directly affect a disease by producing antimicrobials or toxins; or indirectly by competition for space and activation of plant defence systems. Nowadays, there are numerous commercial BCA formulations based on fungi or bacteria to control diseases. For instance, non-pathogenic strains of *Aspergillus flavus* and *F. oxysporum* are used to counteract the pathogenic version of these species. As another example, *Trichoderma* are the active compound of several formulations used to fight *Botrytis cinerea* (Trichodex) or soilborne diseases in general (Root Shield, Plant Shield) [31]. In section 2.4 we will focus on bacterial products, in particular, *Pseudomonas* based formulations [31].

### *2.2.1. Mechanisms of disease suppression.*

Biological control agents can rapidly colonize an environment, thereby preventing the invasion by a pathogen and thus protecting the plant host. To occupy an already colonized niche is very costly in terms of fitness and cell resources. *Pseudomonas* are very good root colonizers that can rapidly spread through the plant roots, preventing the colonization of other pathogens [11]. It was shown that quick colonization of tomato roots by *P. chlororaphis* PCL1391 is crucial for the control of the fungal pathogen *F. oxysporum* [32]. Usually, very good competitors show traits such as phase variation, high motility, utilisation of plant exudates, adhesion to the root, efficient nutrient uptake and siderophores [11]. In some *Pseudomonas* species, phase variation has been related to the introduction of genetic diversity into a population facilitating a rapid reaction to environmental changes [33]. *P. fluorescens* F113 variants with enhanced motility showed improved competitive colonization and biocontrol activity against the fungal and oomycete pathogens, *F. oxysporum* and *P. cactorum* [34]. Furthermore, phase variation

can affect the *gacA/S* system, which controls the production of numerous secondary metabolites, such as siderophores or antimicrobial compounds [33]. Iron uptake using siderophores, such as pyoverdine, is very important to prevent pathogen colonization since iron is essential for microbial growth and a scarce resource in the soil [14, 35]. Siderophores are a very important taxonomic characteristic for fluorescent pseudomonads that inhibit the growth of bacteria and fungi, with fewer potent siderophores in environments with low iron availability [14, 36]. The siderophore pseudobactin from *P. fluorescens* B10 was purified and it was shown to inhibit the growth of *Erwinia carotovora* in vitro assays [37] and *G. graminis var. tritici* and *F. oxysporum* in *in vitro* and greenhouse experiments [38]. Siderophores also increase the iron availability for the plant host contributing to plant growth promotion, but also as chelators to detoxify heavy metal contaminated soils [36].

Besides competitive colonization, bacterial BCAs can produce harmful molecules such as antifungals, reactive oxygen species, cyclic lipopeptides, ACC-deaminases and chitinases involved in pathogen cell wall lysis or volatiles that can stop the progression of different fungal pathogens [22, 39]. Fluorescent *Pseudomonas* produce phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, hydrogen cyanide and cyclic lipopeptides, with demonstrated activity against several root diseases [14, 40]. For example, several antimicrobials produced by the *P. chlororaphis* strains 30-84, O6, PCL1391 or G72 have been related to the control of the fungal pathogens *F. oxysporum* or *G. graminis* [41]. *P. protegens* CHA0 produces 2,4-diacetylphloroglucinol and cyanide that are important in the suppression of the root rot of tobacco caused by *T. basicola* [42], and pyoluteorin which has been shown to supress the damping-off disease produced by *P. ultimum* in cress [43]. Though, a recent study showed that the relative abundance of *Pseudomonas* harbouring antimicrobial genes in agricultural soils only had a weak effect on the

progression of diseases caused by *P. ultimum* and *G. tritici* and that the soil composition had a much stronger impact [16]. Additionally, certain fungal pathogens also possess mechanisms of resistance against BCAs, such as the production of certain secondary metabolite which inhibit antibiotic production in the microbial agents, or detoxification of the antibiotic itself [11].

### *2.2.2. Activation of plant defences*

In the presence of a pathogen, plants also have mechanisms to defend themselves. They can either detect certain microbial compounds such as the bacteria flagellum and the fungal chitin, or endogenous signals derived from the damage caused by enemy invasion. Once the pathogen is detected, the production of reactive oxygen and nitrogen species, kinases, specialized lipids and proteins, and a calcium influx is triggered in the host plant [17, 44]. The specialized pathogens often bypass this microbial pattern-triggered immunity but they still may be detected by the systemic acquired resistance mechanism in the plant which produces attacker-specific effector molecules that prevents diseases. However, plants can also detect the presence of resident root beneficial bacteria which will trigger induce systemic resistance of the plant [17, 22, 29]. This close mutualistic association prepares the plant to better combat a pathogen with a faster or stronger response of the cellular defences upon invasion of a pathogen. For instance, the plants close their stomata faster, reinforce the structural barriers in the plant parts used by the pathogens to invade the host or increase the production of secondary metabolites that damage the pathogens. For example, some pseudomonads such as *P. simiae* WCS417r and *P. putida* WCS358r can trigger systemic resistance in *Arabidopsis thaliana* leading to a more effective defence against *F. oxysporum* by a different metabolic pathway than the simply presence of the pathogen would trigger [17]. Taken together plants have a

complex immune response and rhizobacteria are not only able to suppress soilborne diseases, they also can be effective against diseases of upper plant parts by activating the plants's defence mechanisms.

## 2.3. Biological control of insect pests using bacteria

There are numerous commercial products used against different pest insects based on fungi, bacteria, viruses and nematodes. Fungal-based products are mostly targeting thrips, whiteflies, and aphids, because they can actively go through the insect's chitinous cuticle. Some very commonly used fungi belong to the *Metarhizium* and *Beauveria* genera. Virus formulations currently available on the market are used against lepidopteran species such as *Cydia pomonella*, *Plodia interpunctella*, *Heliothis zea*, *Spodoptera exigua*, *Lymantria dispar*, *Orgyja pseudotsugata*, *Autographa californica* and *Trichoplusia ni* and can remain stable for more than one year under refrigeration. However, in this thesis we will focus on bacterial products that will be further discussed in the following sections.

### *2.3.1. Indirect effects of bacteria against pest insects*

The plant's reaction to damage also applies to tissue destruction caused by herbivorous pest insects. BCAs can increase the plant stress tolerance and trigger its production of defence compounds, enzymes, proteins, secondary metabolites and organic volatiles effective against herbivore insects [17, 22, 29]. For instance, it has been shown that treatment of maize with *B. pumilus* INR-7 strains negatively affects the weight gain of the pest insect *Diabrotica undecimpunctata* [45]. Different mixes of rhizobacteria including *Paenibacillus*, *Bacillus* and *Brevibacillus* species were shown to have an effect on larval weight, development and oviposition of the fall army worm *S. fructiperda* [46].

Rhizobacteria can influence the volatile production of a plant and, as a consequence,

the insect species that are recruited by the host [29, 47]. *A. thaliana* attacked by the leaf-feeder *Mamestra brassicae* and root-colonized by *P. simiae* WCS417r produced more volatiles that attract the parasitic wasp *Microplitis mediator* than *A. thaliana* plants without *M. brassicae* and/or *P. simiae* WCS417r. However, the presence of the beneficial bacterium also increased larval weight of the herbivore [47]. However, increased attraction of hyperparasites is not always the case, as the same bacterial strain induces the production of *A. thaliana* volatiles that are less attractive for the aphid parasitoid *Diaeretiella rapae* [48] or do not have an effect at all on recruiting the parasitic wasp *Cotesia rubecula* [49]. This is an indication of the high specificity of such interactions.

### *2.3.2. Nematode-associated insecticidal bacteria*

One approach to control pest insects in a natural way is the application of entomopathogenic nematodes belonging to the genera *Heterorhabditis* and *Steinenerma*. The nematodes actively enter the insect and release their bacterial symbionts i.e. *Photorhabdus luminescens* and *Xenorhabdus bovienii* [50, 51]. Then, the bacteria rapidly proliferate and produce highly insecticidal compounds thereby killing the insect. Hereafter, the nematode symbiont proliferates by feeding on the bacteria and insect debris [51]. The insecticidal activity of these bacterial species is mainly relying on the MCF (makes caterpillars floppy) toxin encoded by the *mcf* gene. When the bacteria reach the hemocoel, the hemocytes fail to encapsulate them and cannot contain the infection. The Mcf toxin triggers apoptosis in the host immune cells and it is also thought that Mcf triggers apoptosis of the insect midgut cells from the hemocoel side, which leads to a loss in body turgor of the insect causing the floppy phenotype [52, 53]. Other toxins, enzymes, antimicrobials and insecticidal factors have been described in these bacterial species [53, 54]. However, these species lack persistence in the environment and depend on a vector

for infecting the insect host. This makes it difficult to use them independently from their nematode hosts for biocontrol applications [50, 51]. However, several field studies have shown that the inoculation of entomopathogenic nematodes improves the performance and protection of crops, for example of wheat against *Oscinella frit* [55] and of maize against *D. virgifera virgifera* [56]. There are several products based on these nematodes and bacterial symbionts available on the market. Just to name a few examples: Different *Steinenerma* species are used to control the red palm weevil (*Rhynchophorous ferrugineus*), cutworms (*Agrotis* spp., *Spodoptera* spp., *A. gamma*), the codling moth (*C. pomonella*) and sciarid flies. Also, combined formulations of *Steinenerma* and *Heterorhabditis* are used against the black vine weevil *Otiorhynchus sulcatus* (NovAgrica). *Heterorhabditis* strains are used in the product nematop (e-nema) against several weevil species (*Otiorhynchus* spp.). More nematode-based products are currently undergoing the registration process.

2.3.3. *Biocontrol bacteria with oral insecticidal activity*

The most widely used bacterial biocontrol agent against pest insects is *B. thuringiensis*. Most applications, however, do not rely on the viable bacterium itself, but on sprayable products containing their entomopathogenic Cry and Vip toxins and on the construction of transgenic crops expressing these toxins which are especially effective against Lepidoptera pests [50]. The Cry toxins are the most studied and used in agricultural applications due to their high specificity. Once the toxin enters the insect gut, it is activated, binds to specific gut cell receptors and forms pores, which lead to osmotic disequilibrium and cell lysis [57]. Similarly, the parasporal crystals of *Lysinibacillus sphaericus* causes damage in the microvillar epithelial cells of the insect gut. The main targets of commercial applications based on these *L. sphaericus* crystals include

mosquitoes, blackflies and midges. Furthermore, the insecticidal secreted proteins of *B. laterosporus* are highly similar to the Vip toxins of *B. thuringiensis* and have been demonstrated to be toxic to *Diabrotica* spp. [51]. Other non-Bt products are also available and are based on *Burkholderia rinojensis* (Venerate XC against mites) or *P. popillae* (Milky spore powder against Popilla japonica) [58].

The prolonged and extensive use of *B. thuringiensis* (Bt) Cry toxins in modified crops and as fumigants has led to the appearance of resistance in different insect populations worldwide [50, 59, 60]. Insect species, such as *Busseola fusca*, *Diatraea saccharalis*, *D. virgifera virgifera*, *S. frugiperda*, *S. exigua*, *H. virescens*, *Plutella xylostella*, *T. ni*, *Pectinophora gossypiella*, *Helicoverpa zea*, *H. punctigera, Ostrinia furnacalis* and *Striacosta albicosta,* have developed resistance to Bt engineered corn and/or cotton [59–62]. To avoid the appearance of resistance, all insect individuals must be killed and usually, this is not the case. The application of sublethal doses of toxin leads to a rapid appearance of resistance in the population. This was not observed in commercialized Vip crops, so far, and research has shown that a combination of genetically modified crops that produce both Vip and Cry toxins can delay the appearance of resistance. Furthermore, combining Bt and non-Bt plants in the same field leads to a dilution of the resistance genes in the population, which preserves the effectivity of the toxic plants [59]. Several Bt-products are available on the market against caterpillars (XenTrai, Agree WG, Biobit HP, DiPel DF, Javelin WG, Deiver, CoStar, Condor, Crymax), against beetles (boreGONE!, Novodor, Trident) and against mosquitoes as well as flies (AquaBac200/400G/xt/DF3000) [58].

### *2.3.4. The insect immune response to invading bacteria*

Biological control methods based on bacteria with oral insecticidal activity depend on

the direct invasion of the insect gut, which can be a very challenging environment. When the bacteria enter the insect gut, they have to face the already resident gut microbiome which is composed of bacteria, fungi, viruses and protozoa [63, 64]. The insect system can detect an anomalous increase in certain particles, such as the proteoglycans of the bacterial surface, and trigger a response through the IMD and DUOX systems. This activates the release of antimicrobial peptides and reactive oxygen and nitrogen species, respectively. If the invading bacterial pathogens manage to persist in the gut, they have to break through the peritrophic matrix, a highly hydrophilic mucus matrix rich in chitin microfibrils, proteins, glycoproteins and proteoglycans, which makes it very resistant to penetration by physical forces [65, 66]. Once in the hemocoel, pathogens have to survive the intervention of the cellular immune response of the insect. In this thesis, we focus on the Lepidoptera order for which different kinds of hemocytes i.e. granulocytes, plasmatocytes and oenocytoids play different roles in the response against pathogens. Plasmatocytes encapsulate the bacterial invaders, while granulocytes phagocyte the pathogens. Oenocytoids control the production of melanin, which will coat the bacteria hindering their nutrient uptake [67]. The cells of the fat body also trigger the Toll response which leads to the production of antimicrobial peptides that challenge pathogens in the hemolymph (Fig. 2) [63, 68, 69]. A successful pathogen infection can only occur if the commensal bacteria and immune system fail to repel the invaders as shown for human microbiota and the *P. aeruginosa* model [70].

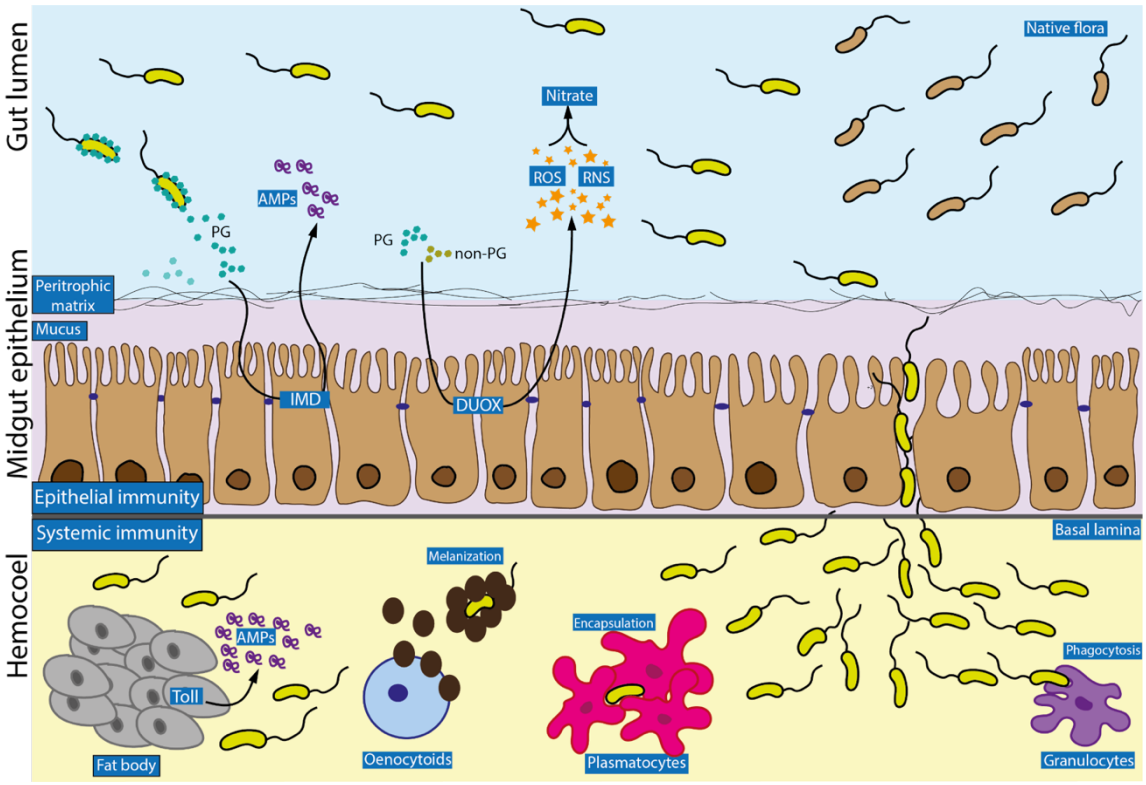


**Figure 2. Bacterial gut invasion and milestones for a successful infection.** Microbial pathogens have to fight against the resident microflora to persist in the gut. The insect can detect the presence of proteoglycans (PG) from the cell wall triggering the lMD and DUOX immune response which produces antimicrobial peptides (AMPs) and reactive oxygen and nitrogen species (ROS, RNS), respectively. Afterwards, the bacteria need to break through the peritrophic matrix in order to reach the hemocoel where they face the cellular immune response of the insect composed by oenocytoids (production of melanin), plasmatocytes (encapsulation), granulocytes (phagocytosis) and the fat body (production of AMPs). Green bacterial cells are the invading bacteria, brown bacterial cells the resident gut microflora and blue boxes represent the insect defences.

### *2.3.5. Insecticidal Pseudomonas*

The first pathogenic interaction between a pseudomonad and an insect was described for an unknown *P. aeruginosa* strain and *Melanoplus bivittatus* in 1957, but no responsible toxin or factor was identified [71, 72]. Since then, numerous *Pseudomonas* strains belonging to different species have been described as insecticidal and several toxins and

other mechanisms contributing to insect pathogenicity, that seem to be characteristic for each species, have been described [72]. One of the most widely known entomopathogenic pseudomonads is *P. entomophila* L48, isolated from a *Drosophila melanogaster* female. The insecticidal activity of *P. entomophila* L48 has been related to the pore-forming toxin monalysin, cyclic lipopeptides and the metalloprotease AprA [73–76]. On the other hand, in *P. aeruginosa* the type III secretion system and effectors used to infect humans, seem to be also effective against insects [72].

This thesis is specially focused on plant beneficial and insecticidal fluorescent pseudomonads belonging to the *P. chlororaphi*s and *P. protegens* species. These bacterial species are able to invade the challenging insect gut, transmigrate into the hemocoel, and cause a systemic infection that will ultimately lead to the death of the insect host [77, 78]. Twelve years ago, a very similar gene to the *mcf* toxin of *P. luminescens* and *X. nematophila* was found in the genomes of *P. protegens* CHA0 and Pf-5, the Fit toxin (fluorescent *Pseudomonas* insecticidal toxin) encoded by the *fitD* gene [77]. The Fit toxin was partially related to the insecticidal activity of *P. protegens* CHA0 and other *P. protegens* and *P. chlororaphis* strains, but it was not the only insecticidal trait in this bacterial species [79].

In the last few years, several other factors such as the antimicrobial hydrogen cyanide [80], the cyclic lipopeptides orfamide [80, 81], sessilin [80] and Clp1391 [80], the type VI secretion system [82], a chitinase and a phospholipase [83], specific lipopolysaccharide O-antigens [84] and the toxins rhizoxin [85] and IPD072Aa [86] were related to insecticidal activity in *P. protegens* and *P. chlororaphis,* which was redefined as a multifactorial trait. However, it is still unknown where, when and how those metabolites and factors act during the bacterial invasion and the disease progression. We address this a question in detail in chapter 3.

Moreover, it is still cryptic what the real relationship is that these well-known root colonizing bacteria establish with insect hosts. It is also unknown whether they are natural commensals of these animals, or if they had evolved to adapt, colonize and kill the insects as an opportunistic pathogen and to use them as nutrient source. Root colonizing bacteria could also be both commensals and opportunistic pathogens in a species-specific interaction. *Pseudomonas* have been found associated with different arthropods in the past [87], but very few studies focus on agricultural environments. These questions will be addressed in more detail in chapters 2 and 4.

## 2.4. Use of *Pseudomonas* as plant growth promoters and microbial control agents

Plant growth promoting rhizobacteria and microbial control agents can be applied as biofertilizers, individually or in consortia, in order to increase crop yield as a response to the food crisis. Plant growth promotion and biocontrol are very closely related because they share several factors and that is why microbes with such dual activity are of vital importance in agricultural biotechnology. The use of plant growth promoting microorganisms will lead to reduce the use of chemical fertilizers and pesticides, which have negative impacts on water sources and human health [9, 10, 15]. Among bacterial promoters, the *Pseudomonas* genus has gained a lot of attention because of its duality i.e. as growth promoters and biocontrol agents, of not only soilborne pathogens, but also of pest insects, as was recently discovered. This dual activity makes the *Pseudomonas* genus very attractive as an alternative to traditional microbial pesticides. Pesticide resistance is rising due to overexploitation and bad agricultural practices. However, *Pseudomonas* strains pose a challenge regarding their preservation and application, as products lose viability after several weeks in contrast to spore-forming *Bacillus* spp. formulations [14].

Different *Pseudomonas* strains belonging to the *P. fluorescens*, *P. protegens*, *P.*

*chlororaphis*, *P. putida* and *P. simiae* have been tested alone or in combination with *Bacillus* spp. or other organisms using different formulations and against different pest insects in different crops [45, 55]. For example, *P. protegens* CHA0 and *P. chlororaphis* PCL1391 were inoculated in a wheat field attacked by *O. frit.* The colonization of wheat roots by these strains had a positive effect on the resistance of the plants against the insect attack [55]. A similar effect was observed in a maize field trial with both bacterial species against *D. virgifera virgifera* [56]. However, very few *Pseudomonas* biocontrol and plant growth promotion formulations are available on the market [31, 58] and the few products that are commercially available are only registered as biofertilizers or as biocontrol agents against fungal diseases but not against insects. *P. chlororaphis*-based products are being used against *Pythium* spp., *R. solani* and *F. oxysporum* in vegetables and ornamental plants (AtEze, Cedomon, Cerall). Different pseudomonads strains, such as *Pseudomonas* sp. DSMZ 13134 (Proradix), are also used for such soil borne diseases. Other pathogens, such as *Sclerotinia homeocarpa, Colletotrichum graminicola, P. aphanadermatum* and *Michrodocium nivale,* are being controlled with *P. aureofaciens* strain Tx-1 (Bio-Jet, Spot-Less). Furthermore*, P. fluorescens* A506 is part of BlighBan A506 and Frostban formulations which are used to treat frost damage, fireblight caused by *E. amylovora* and the bunch rot caused by *B. cinerea*. Postharvest diseases are also of high concern in agriculture and *P. syringae* ESC-10 is the biocontrol agent of the Bio-Save 10LP used to prevent the emergence of such diseases [31].

## 3. Aims of the thesis and thesis outline

Over the past few years, the two multi-talented species *P. chlororaphis* and *P. protegens* have gained increasing attention because of their ability to colonize plant and insect hosts. Furthermore, these species have antagonistic effects against plant pathogens and insect pests. Recent research has related insecticidal activity to numerous factors, but it is still widely unknown how insect invasion and pathogenesis occur, and what specific set of factors are required for colonizing an insect compared to colonizing a plant root. Once insecticidal activity was discovered, it was explored mainly using model strains e.g. *P. chlororaphis* subsp. *piscium* strain PCL1391 and *P. protegens* strains CHA0 and Pf-5. Interestingly, these model strains did not have the same insecticidal effect on all the insect species they were tested on. This raised several questions: Can *P. chlororaphis* and *P. protegens* establish different kinds of relations with insects e.g. pathogenic and commensal interactions? Is there a specificity for certain insect species or order and is there a specialization within the two species for plant or insect hosts? Additionally, since most of the research on insecticidal pseudomonads was performed with strains isolated from soil or from plants, it is unknown if insecticidal *Pseudomonas* are naturally associated with arthropods. Based on these knowledge gaps, three major research topics were addressed in this thesis:

1. *Exploration of different Pseudomonas-insect relationships*

   Based on the observation that model *P. protegens* and *P. chlororaphis* strains do not have the same infectivity for all studied insects, the question arises whether non- or less lethal interactions are due to the lack of ability to colonize certain insect species, or whether the bacteria can establish commensal interactions with insects. This question was addressed in **chapter 2**. *P. xylostella*, *Pieris brassicae*,

*Delia radicum* and *O. sulcatus* larvae were fed with *P. protegens* CHA0 and larval mortality, as well as bacterial colonization in larvae, pupae and adults was assessed. While *O. sulcatus* and *D. radicum* larvae were largely resistant to CHA0, the mortality of *P. xylostella* and *P. brassicae* depended on the bacterial doses and larval age. Even though insecticidal effects caused by CHA0 were not homogeneous among the four species, bacteria persisted in all of them across the different insect life-stages from larva to adult. Additionally, anomalous morphologies were observed for a part of the *P. brassicae*, *P. xylostella* and *D. radicum* adults emerging from treated larvae. Finally, we could demonstrate that healthy looking *D. radicum* adults still carry bacteria and are able to transmit them to a new host plant in a gnotobiotic system. The findings of **chapter 2** indicate that *P. protegens* CHA0 can either cause lethal infections in larvae and use insects as food source or persists in the insect throughout the different development stages and use adults as vectors for dispersal.

2. *Unravelling the process of insect pathogenesis and the molecular patterns of insect invasion compared to plant-root colonization*

   At the start of this thesis, it was still unknown how invading an insect differs from colonizing a plant-root and what specific set of tools the bacteria use to conquer these different hosts. Besides, it was not clear when and how already described insecticidal factors play their role during insect invasion and killing. Furthermore, we expected that there are additional, yet unidentified protein/factors involved in this process. In **chapter 3**, we addressed these gaps using comparative transcriptomics. *P. protegens* CHA0 was fed to *P. xylostella*, injected to *Galleria mellonella* and inoculated on wheat roots. Samples were collected and RNA was extracted and subjected to NextSeq Illumina sequencing. The analysis revealed the

time points during pathogenesis where known factors are expressed, and allowed to discover new potentially insecticidal traits. Among the newly discovered traits, effector proteins/toxins released by two-partner secretion systems were shown to be important during insect pathogenesis in *P. protegens* CHA0 using a mutational approach. In addition, the presence/absence of the studied factors in other pseudomonas species was investigated by an orthologue analysis. Altogether, the data collected from the RNA-seq led to the proposal of a comprehensive insect pathogenesis model in *Pseudomonas*.

3. *Insects as an alternative Pseudomonas host*

   *Pseudomonas* of the *protegens* and *chlororaphis* species possess numerous insecticidal factors. However, their activity against insects was, so far, mostly studied with strains isolated from soil or plants and the natural relationship with these animals remained unknown. In **chapter 4** we had two aims: 1) to search for insecticidal pseudomonads inside insects and other arthropods and 2) to compare plant and insect isolates in order to investigate whether there is a certain host adaptation within the two *Pseudomonas* species. Since, regarding biological control, the activity against agricultural pest is of major importance, we decided to collect arthropods, soil and roots from a wheat field, a potato field and a nearby undisturbed grassland in two consecutive years. Insecticidal pseudomonads belonging to the *P. chlororaphis* and *P. protegens* species could be isolated from different arthropod orders, e.g. insects and myriapods and a collection of animal and plant/soil isolates was characterized for plant- and insect interactions. Interestingly, no matter whether they were isolated from plants or from arthropods, all isolates were able to colonize cucumber roots and protect them against an oomycete pathogen, but also to colonize *P. xylostella* larvae and to cause lethal

infections. However, the insecticidal capabilities of *P. chlororaphis* isolates were more variable than that of its sister species *P. protegens*. Two *P. chlororaphis* Coleoptera isolates with reduced insecticidal capabilities were examined more in detail. Their genomes were compared to the closely related, but highly insecticidal *P. chlororaphis* subsp. *piscium* PCL1391 using a single nucleotide polymorphism analysis. The analysis revealed several mutations in genes encoding known insecticidal factors, that could be the cause for delayed killing speed and efficiency of these strains. Altogether the results of **chapter 4** indicate that *P. chlororaphis* and *P. protegens* are commonly associated with healthy insects and point towards a certain adaption to different insect hosts, especially within the *P. chlororaphis* species.

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# Chapter 2

Persistence of root-colonizing *Pseudomonas protegens* in herbivorous insects throughout different developmental stages and dispersal to new host plants

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## 1. Abstract

The discovery of insecticidal activity in root-colonizing pseudomonads, best-known for their plant-beneficial effects, raised fundamental questions about the ecological relevance of insects as alternative hosts for these bacteria. Since soil bacteria are limited in their inherent abilities of dispersal, insects as vectors might be welcome vehicles to overcome large distances. Here, we report on the transmission of the root-colonizing, plant-beneficial and insecticidal bacterium *Pseudomonas protegens* CHA0 from root to root by the cabbage root fly, *Delia radicum*. Following ingestion by root feeding *D. radicum* larvae, CHA0 persisted inside the insect until the pupal and adult stages. The emerging flies were then able to transmit CHA0 to a new plant host initiating bacterial colonization of the roots. CHA0 did not reduce root damages caused by *D. radicum* and had only small effects on *Delia* development suggesting a rather commensal than pathogenic relationship. Interestingly, when the bacterium was fed to two highly susceptible lepidopteran species, most of the insects died, but CHA0 could persist throughout different life stages in surviving individuals. In summary, this study investigated for the first time the interaction of *P. protegens* CHA0 and related strains with an insect present in their rhizosphere habitat. Our results suggest that plant-

colonizing pseudomonads have different strategies for interaction with insects. They either cause lethal infections and use insects as food source or they live inside insect hosts without causing obvious damages and might use insects as vectors for dispersal, which implies a greater ecological versatility of these bacteria than previously thought.

## 2. Introduction

Every year worldwide crop production is facing major harvest losses due to plant pathogens and pest insects. Belowground attackers are especially difficult to tackle with chemical pesticides and adverse environmental effects of these products demand for alternative strategies such as the use of antagonistic organisms to control pest organisms, known as biological control. Root-colonizing bacteria of the *Pseudomonas fluorescens* species complex [1] have been extensively studied for their beneficial effects on plants, e.g. the suppression of root diseases and the promotion of plant growth [2, 3], and this research already led to several commercial products [4].

Within the *P. fluorescens* species complex there is a specific phylogenetic group, the *Pseudomonas chlororaphis* subgroup [5], comprising multi-talented bacteria of special interest for use in crop protection. These bacteria have, in addition to the plant-beneficial activity, features to colonize insects as an alternative host [6–9]. Strains of the *P. chlororaphis* subgroup were even found to exhibit potent oral activity against larvae of Lepidoptera [6, 8, 10] as well as against *Drosophila melanogaster* [11] and a *P. chlororaphis* toxin was found to be active against the western corn rootworm *Diabrotica virgifera virgifera* [12]. Several factors have been identified to contribute to insect pathogenicity: the Fit toxin, antimicrobial metabolites, secreted enzymes, lipopolysaccharide O antigen and the insecticidal protein IPD072Aa [6, 9–19]. In-depth studies on the Fit toxin in the model strain *P. protegens* CHA0 revealed that the bacteria produce this insecticidal protein specifically in insects, but not on plant roots [20, 21]. Accordingly, the bacteria seem to sense their environment and regulate the production of specific compounds depending on the specific needs in the encountered habitat.

Although there is a growing body of evidence that insects represent an alternative host for *P. chlororaphis* subgroup bacteria, the ecology of their insect-associated lifestyle is still elusive. To date, oral insecticidal activity has been investigated only in model insects feeding

on leaves [6, 8, 10, 14, 20]. However, many known strains of the *P. chlororaphis* subgroup are rhizosphere isolates, e.g. *P. protegens* CHA0 was isolated from tobacco roots, and their interaction with root-feeding insects is therefore of much greater ecological relevance. It is still unknown whether these bacteria are also pathogenic to soil insects and therefore have a potential as biocontrol organisms of root pests. Furthermore, the discovery that several plant-beneficial pseudomonads exhibit specific adaptions to a life in insects [7, 16, 17, 20] raised the hypothesis that insects might represent attractive vectors to reach new plant hosts. Rhizobacteria are limited in their inherent dispersal abilities and may largely depend on passive transport, such as water flows, to overcome large distances. Alternatively, dispersal by means of a vector is a plausible manner of attaining new habitats. Insect-mediated dispersal has been described for several plant-pathogenic bacteria [22], but data on transmission of beneficial rhizobacteria to a new host plant is scarce. *Pseudomonas chlororaphis* L11, an efficient root colonizer without known biocontrol activity, was found to be transmitted from plant to plant by the red-legged grasshopper, *Melanoplus femurrubrum*, as well as by the southern corn rootworm, *Diabrotica undecimpunctata* susp. *howardii* [23, 24], but mainly when insect vectors were feeding on L11-infested foliage. In contrast to L11, which moves from the rhizosphere into the foliage [25], *P. protegens* CHA0 was not found to move to above ground plant parts [26, 27]. For the dispersal of a rhizobacterium, which is restricted to below-ground plant parts, an insect with a root-feeding larval and an above-ground flying adult stage would represent a suitable vector. A prerequisite for this kind of dispersal is persistence of the bacteria in the insect host and transstadial transmission from larva over pupa to the adult stage.

This study investigates the interaction of *P. protegens* CHA0 with a root-feeding pest insect, the cabbage root fly *Delia radicum*. Their larvae feed on brassicaceous plants, pupate in the soil and emerging adults fly to a new host plant to deposit eggs. Our first aim was to assess the oral activity of different *P. chlororaphis* subgroup strains against this insect. While

all *P. chlororaphis* subgroup strains tested so far are, to a high degree, lethal to many lepidopteran insect species [6, 8], we found that the survival of *D. radicum* larvae was affected by certain strains of the *P. chlororaphis* subgroup, but not by *P. protegens* CHA0. The second aim was to compare the ability of model strain CHA0 to persist in non susceptible and highly susceptible insects. CHA0 was able to persist throughout different life-stages in all tested insect species independently of their susceptibility level indicating that this is a rather general phenomenon. For *D. radicum*, we could show in addition that adult flies emerging from larvae that fed on CHA0 colonized roots transmitted the bacteria to the roots of new host plants. This provides the first direct evidence for the possibility of insect-mediated dispersal of *P. protegens* CHA0. Overall, our data indicate that indeed insects might be both, relevant alternative hosts and vectors for certain plant-beneficial rhizobacteria.

# 3. Materials and Methods

## 10.1. Bacterial cultures

The bacteria used in this study are listed in Table 1. Strains with a constitutively expressed GFP tag were generated by means of the Tn7 delivery vectors pBK-miniTn*7-gfp1* or pBKminiTn*7-gfp2* [20]. In the results and discussion sections we always use wild-type names of strains. Whether GFP-tagged variants were used is indicated in the materials and methods and in the figure legends. GFP-tagged strains did not differ in their activity from the respective wild-type strains. Bacteria were cultured in lysogeny broth (LB), supplemented with either kanamycin (25 µg/ml) or gentamicin (10 µg/ml) for GFP expressing strains, overnight at 24 °C and 180 rpm. For the cauliflower experiments with *D. radicum* 200 µl of LB cultures were used to inoculate King's B (KB) agar plates [28] supplemented with gentamicin (10 µg $ml^{-1}$). After one day, bacterial cultures were scraped off the plates, suspended, washed twice in sterile $ddH_2O$, and $OD_{600}$ was adjusted to the desired concentration ($OD_{600}$ of 0.125 corresponds to about $10^8$ cfu/ml). In all other experiments, LB cultures were washed twice (once for radish experiments) in sterile 0.9% NaCl solution or water before adding cell suspensions adjusted to the desired concentration to diets or roots/radish, respectively.

**Table 1. Bacterial strains used in this study**

| Strain | Genotype, phenotype or relevant characteristics | Insecticidal activity | Reference or source |
|---|---|---|---|
| *Pseudomonas protegens* CHA0 | Wild type, isolated from tobacco roots | Yes | [29, 30] |
| *Pseudomonas protegens* CHA0-*gfp2* | CHA0::*att*Tn*7-gfp2;* $Gm^r$ | Yes | [21] |
| *Pseudomonas protegens* CHA1176 | CHA0::*att*Tn*7-gfp2 fitD-mcherry;* $Gm^r$ | Yes | [21] |
| *Pseudomonas protegens* PF-*gfp2* | PF::*att*Tn*7-gfp2;* $Gm^r$ | Yes | This study, for wild type PF see [31] |
| *Pseudomonas* sp. CMR12a-*gfp1* | CMR12a::*att*Tn*7-gfp1;* $Km^r$ | Yes | This study, for wild type CMR12a see [32] |
| *Pseudomonas chlororaphis* PCL1391-*gfp2* | PCL1391::*att*Tn*7-gfp2;* $Gm^r$ | Yes | This study, for wild type PCL1391 see [33] |
| *Pseudomonas thivervalensis* PITR2-*gfp2* | PITR2::*att*Tn*7-gfp2;* $Gm^r$ | No | This study, for wild type PITR2 see [34] |

$Gm^r$, gentamicin resistance; $Km^r$, kanamycin resistance

## 10.2. Radish experiment for testing the susceptibility of *D. radicum* to different *Pseudomonas* strains

*D. radicum* was reared as described by Razinger et al. [35], but larvae were fed on turnip cabbage instead of rutabaga. Greens of organically grown radishes (Migros, Switzerland) were cut off about 0.5 cm above the bulbs. Bulbs were then washed with tap water and 70% ethanol, dried with household paper and submerged for 10 min in a bacterial suspension of $OD_{600}$ of 0.47 or $ddH_2O$ as a control. Then the radishes were buried in pots (345x276x80 mm) (Bachmann Plantec AG, Switzerland) filled with sterile quartz sand. Six to eight eggs of *D. radicum* were deposited on top of the sand and the pots covered with aluminium foil were incubated in a climate chamber (16-h day, 20°C, 210 µmol $m^{-2}$ $s^{-1}$; 8-h night cycle, 18°C) for four weeks. Developing pupae were harvested by washing the sand over a sieve. All pupae emerging from one pot were photographed together and pupal size was measured by means of an ImageJ macro. Two weeks later, flies emerging from

pupae were quantified. Flies of the CHA0- *gfp2* and control treatments were checked for presence of CHA0 by incubating entire flies each in 1 ml LB supplemented with chloramphenicol (13 µg $ml^{-1}$), cycloheximide (100 µg $ml^{-1}$) and gentamicin (10 µg $ml^{-1}$) for two days. To verify the identity of the growing bacteria, they were checked for GFP expression under a Leica DM2500 microscope (Leica Microsystems CMS GmH, Wetzlar, Germany). The experiment was conducted twice.

### 10.3. Persistence/transmission of *P. protegens* CHA0 in experiments with cauliflower/rapeseed and *D. radicum*

This experiment aimed at investigating the ability of *P. protegens* CHA0 to persist in *D. radicum* throughout different life stages and the possibility of flies vectoring the bacterium from one host plant to another. Cauliflower (original host) and rapeseed (new host) were chosen for the experiments since in nature *D. radicum* might also move between these two brassicacean crop plants during its life-cycle. Cauliflower plants (four trays each containing twelve pots, one plant per pot) were grown with and without (control) *P. protegens* CHA0-*gfp2* for three weeks as detailed in the Supplementary Methods. Then, five freshly hatched *D. radicum* larvae were added to each plant. Four weeks later, plant shoots were weighed and root systems were washed on a sieve to collect pupae as well as non-pupated larvae. Larvae were directly extracted for bacteria monitoring while pupae were kept in Petri dishes until further processing. Bacterial root colonization was assessed as described in Supplementary Methods. Pupal size was measured as described for the radish experiments. Ten to twelve pupae per treatment were extracted to assess colonization by inoculant bacteria as described below, three to four pupae were transferred to each of the transmission microcosms and the remaining pupae (44-56 per treatment) were observed to determine the rate of fly emergence. The transmission microcosms were designed to test whether flies are

transmitting CHA0 to the roots of a new host plant (rapeseed) and are described in detail in the Supplementary Methods. Briefly, each transmission microcosm consisted of four rapeseed plants grown axenically on a sand-vermiculite substrate in closed plastic beakers. Little containers with three to four pupae, coming either from control or *P. protegens* CHA0-*gfp2* treatments of the cauliflower experiment, were added to each transmission microcosm. The containers were used to prevent direct contact of the pupae with the plant and the substrate, but were open at the top to allow the flies to escape into the transmission microcosms. Nine days after flies had started to emerge and fly around inside the microcosms, roots of rapeseed plants were checked for colonization by *P. protegens* CHA0-*gfp2* as described for cauliflower plants (Supplementary Methods). Roots of plants grown in the same beaker were pooled for analysis.

### 10.4. Oral toxicity of *P. protegens* CHA0 toward *Plutella xylostella* and *Pieris brassicae* and persistence during the insect life cycle

The experiments with *P. xylostella* were conducted as detailed in Flury et al. [14] and are briefly described in the Supplementary Methods. *P. brassicae* larvae were reared at 25°C, 60% relative humidity and a 16-h day, 8-h night cycle and fed with Brussels sprouts variety Topline F1. During the experiments larvae were kept individually in Petri dishes lined with a moisturized filter paper and were fed with a pellet of artificial diet [36] inoculated with 10 µl of suspension of *P. protegens* CHA0-gfp2 or CHA0 cells at an $OD_{600}$ of 20 or amended with sterile 0.9% NaCl solution (control). Larvae that did not consume the entire diet pellet were excluded from the experiment. After 24 h, larvae were transferred in groups of six into 720 ml Pint-sized BugDorms (BugDorm, Taiwan) and fed with cabbage until pupation. 24 - 32 larvae per treatment were used for monitoring mortality. Larvae and pupae were considered dead when they did not react to poking. Further individuals (alive, crippled and dead) of each

developmental stage were assessed for bacterial colonization as described below. The experiment was conducted three times.

### 10.5. Assessment of bacterial colonization in insects

To assess bacterial colonization, insects derived from the different experiments described above were surface sterilized (20 s 70% ethanol, 20 s sterile dd$H_2O$ for *P. xylostella* and *P. brassicae*; 20 s 0.05% SDS, 20 s 70% ethanol, 20 s sterile dd$H_2O$ for *D. radicum*) and then homogenized in sterile 0.9% NaCl solution with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). Efficacy of insect surface sterilization was tested as detailed in the Supplementary Methods. Dead individuals were extracted within 24 h after death occurred. The resulting suspensions were serially diluted in sterile 0.9% NaCl solution and plated onto KB agar plates supplemented with chloramphenicol (13 µg $ml^{-1}$), cycloheximide (100 µg $ml^{-1}$) and gentamicin (10 µg $ml^{-1}$). For *D. radicum*, plates were additionally supplemented with ampicillin (40 µg $ml^{-1}$). GFP-expression of growing colonies was verified under the microscope (ex: 480/BP 40 nm, em: 527/BP 30 nm).

### 10.6. Microscopy

Microscopic investigations and sample preparation of *P. xylostella* larvae are described in Supplementary Methods.

### 10.7. Statistics

Data analysis was performed in RStudio version 0.98.1017 (http://www.rstudio.com) using R version 3.1.2. Data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance and according to the results a Student's t test or a Mann-Whitney *U* test (cauliflower experiments) or a Kruskal Wallis (radish experiment) was performed. For

*Pieris* experiments, the log-rank test of the Survival package of R was used to compare survival curves.

# 4. Results

## 10.1. Minor effects of *P. chlororaphis* subgroup bacteria on the root pest *D. radicum*

To investigate how bacteria of the *P. chlororaphis* subgroup, known for their insecticidal activity against various leaf-feeding insects [8, 10, 14], interact with an insect living in their natural habitat, the rhizosphere, we tested the impact oral activity of these bacteria on the cabbage fly *D. radicum* following oral infection, an important root pest on brassicaceous crops.

In an experiment with bacteria-treated radish bulbs, aiming at comparing different bacterial strains, *P. chlororaphis* PCL1391 caused a significant reduction of the pupation rate (Fig. 1A) and the pupal size (Fig. 1C) compared to the control. Moreover, in this experiment a reduction in pupal size was also observed for the strains *P. protegens* CHA0 and *Pseudomonas* sp. CMR12a (Fig. 1C). However, in a repetition of the experiment only *Pseudomonas* sp. CMR12a caused a significant reduction in the pupation rate (Fig. 1B) and the pupal size (Fig. 1D). In a further experiment, larvae of *D. radicum* were feeding on roots of cauliflower plants inoculated with or without *P. protegens* CHA0 (Fig. 2). *P. protegens* CHA0 developed on average population sizes of 6.51 ± 0.59 (experiment 1) and 5.92 ± 0.56 (experiment 2) $\log_{10}$ cfu per g of root fresh-weight. In both experiments, no significant differences in pupation rate, pupal size and in the number of flies emerging from pupae could be detected between control and CHA0 treatments (Fig. 2A, Supplementary Figure S1, Supplementary Figure S2A, B). Moreover, shoot weights from plants inoculated with CHA0 did not significantly differ from those of control plants (Supplementary Figure S2C, D).

Overall, some *P. chlororaphis* subgroup bacteria, particularly *Pseudomonas* sp. CMR12a and *P. chlororaphis* PCL1391, seem to affect the performance of *Delia* larvae leading to smaller and fewer pupae, but the effects are rather moderate and variation is high.

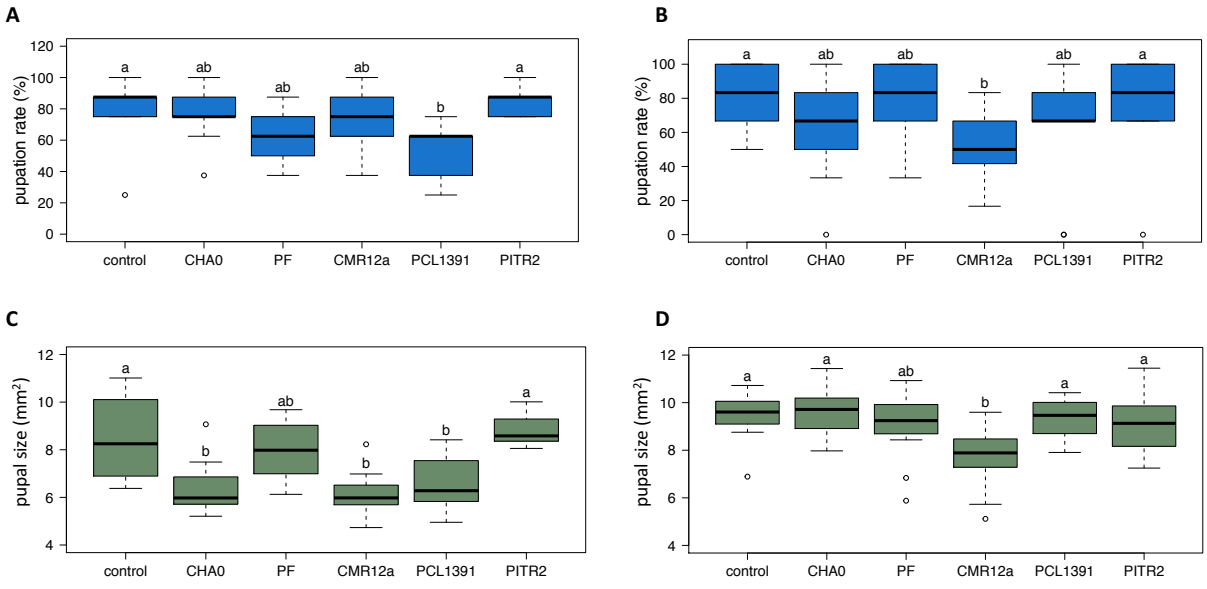


**Figure 1. Certain strains of the *Pseudomonas chlororaphis* subgroup negatively affect *Delia radicum* pupation rate and pupal size.** Pupation rate per egg **(A, B)** and pupal size **(C, D)** of *D. radicum* larvae fed on radishes inoculated with strains CHA0, PF, CMR12A, or PCL1391 of the *P. chlororaphis* subgroup, known to have insecticidal activity, or with the non-insecticidal strain PITR2 or amended with water (controls). In experiment one **(A, C)**, nine radishes per treatment were infested each with eight *D. radicum* eggs, while in experiment two **(B, D)** twelve radishes per treatment were infested each with six eggs. Treatments with different letters significantly differed from each other (Kruskal-Wallis, $p<0.05$). Control, sterile water; CHA0, *Pseudomonas protegens* CHA0-*gfp2*; PF, *Pseudomonas protegens* PF-*gfp2*; CMR12a, *Pseudomonas* sp. CMR12a-*gfp1*; PCL1391, *Pseudomonas chlororaphis* PCL1391-*gfp2*; PITR2, *Pseudomonas thivervalensis* PITR2-*gfp2*.

## 10.2. Transstadial transmission of *P. protegens* CHA0 in *D. radicum* and dispersal to new host plants by adult flies.

In a previous study, we reported that certain strains of the *P. fluorescens* species complex that are not causing fatal infections are still able to persist inside larvae of the cotton leafworm *Spodoptera littoralis* [6]. Since no effect on survival of *D. radicum* larvae was observed in the present study, we were wondering whether *P. protegens* CHA0 was able to establish a rather commensal interaction allowing the bacterium to colonize the larvae, to persist inside the insect throughout different life stages and eventually to be transferred to new host plants by adult flies.

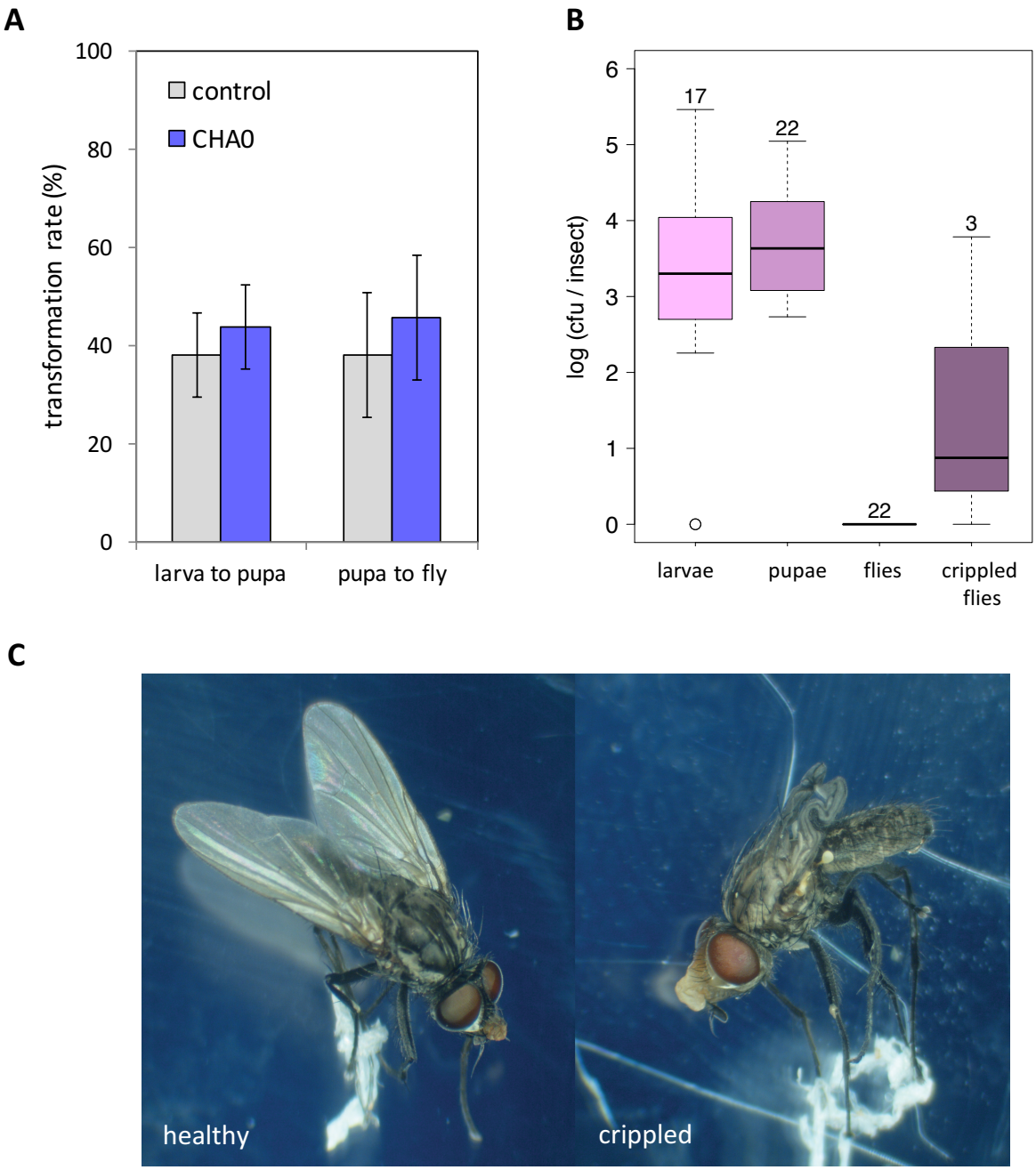


**Figure 2. *Pseudomonas protegens* CHA0-*gfp2* does not affect survival of *Delia radicum*, but it persists throughout different life stages.** Five freshly hatched *D. radicum* larvae were added to cauliflower plants (four trays per treatment, each containing twelve pots) grown with *P. protegens* CHA0-*gfp2* (CHA0) on the roots or without (control). **(A)** The pupation rate and the rate of flies emerging from pupae did not significantly differ between the control and the CHA0 treatment ($p < 0.05$; Mann-Whitney *U* test). Error bars depict standard deviations of the means of replicate trays. A repetition of the experiment is shown in Supplementary Figure S1. **(B)** Population sizes of *P. protegens* CHA0-*gfp2* detected in *D. radicum* at different life-stages after larvae fed on roots colonized by *P. protegens* CHA0-*gfp2*. No CHA0 was detected in individuals emerging from the control treatment (data not shown). Data are pooled from two experiments. Numbers above boxes indicate sample size. **(C)** Some flies in the CHA0 treatment exhibited morphological defects, e.g. crippled wings.

**Persistence from larval to adult stage.** Extraction of Delia larvae and pupae from the two cauliflower experiments showed that they indeed were colonized by CHA0 (Fig. 2B) at average levels of 2.7 $\log_{10}$ and 3.8 $\log_{10}$ cfu per insect. No CHA0 was detected in insects from the control treatment. In contrast to results on larvae and pupae, our method of homogenizing the flies and plating serial dilutions generally revealed no *P. protegens* CHA0 associated with adult flies whether these were surface-sterilized or not. However, a few flies that emerged from CHA0 treated roots showed developmental defects, most obviously malformations of the wings (Fig. 2C). Three crippled flies were extracted and remarkably two of them were colonized by CHA0 (Fig. 2B). Since healthy looking flies from the cauliflower experiments were able to transmit CHA0 to a new host plant (see results below), they must have carried the bacteria although we did not detect them. Therefore, we assessed larger amounts of *Delia* flies for presence of CHA0 by a second method. Entire flies emerging from control and CHA0 treatments of radish experiments were placed in selective liquid medium without prior surface disinfestation and the medium was then checked for growth of CHA0. This qualitative approach revealed that in one experiment 76% (n=21) and in the other experiment 53% (n=30) of the healthy flies were carrying CHA0. No CHA0 was detected on flies from the control treatment.

**Dispersal by flies to a new host plant.** To assess, whether *Delia* flies which had been exposed to *P. protegens* CHA0 at the larval stage are able to transmit CHA0 to a new host plant, we elaborated a specific test system. Pupae that emerged from control and CHA0 treatments in the cauliflower experiments were transferred into closed plastic beakers (three to four pupae per beaker) containing rapeseed plantlets grown axenically on a sand-vermiculite substrate. To avoid transmission of bacteria by the pupae, those were kept in containments preventing direct contact with plants or substrate. Emerging flies were flying around in the transmission microcosms and in several of them they also

laid eggs. Nine days after first flies started to emerge, root systems were assessed for bacterial colonization. The roots of twelve out of thirteen (experiment one) and of eight out of nine (experiment two) transmission microcosms, which had been exposed to flies emerging from the CHA0 treatment, were indeed colonized by *P. protegens* CHA0 (Fig. 3). In both experiments, no CHA0 could be detected on roots from transmission microcosms that had been exposed to control flies (Fig. 3). In microcosms with successful CHA0 transmission, average colonization rates in experiments one and two were 5.0 $\log_{10}$ and 4.3 $\log_{10}$ cfu per g of roots, respectively. Hence, *Delia* flies were able to transmit *P. protegens* CHA0 to a new host plant.

In summary, we provide first evidence that *P. protegens* CHA0 when ingested by larvae can be transstadially transmitted not only to the pupal stage, but even to the adult stage and that adult insects can disperse the bacterium to new host plants.

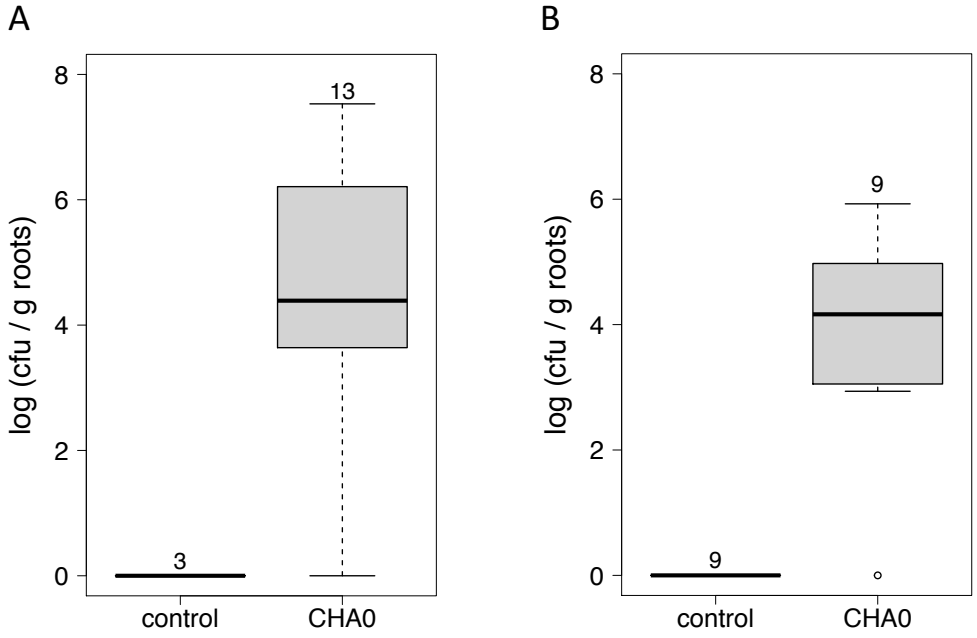


**Figure 3. *Pseudomonas protegens* CHA0 can be dispersed to new host plants by adult *Delia radicum* flies.** Colonization levels of CHA0-*gfp2* on roots of rapeseed plants. Plants were grown in sterile soil for 18 days and were then exposed for nine days to flies, which had developed from larvae feeding on roots of control or CHA0-*gfp2* treated cauliflower plants, i.e. the previous plant host. In the CHA0 treatment, in twelve out of thirteen (experiment one) **(A)** and in eight out of nine (experiment two) **(B)** systems, rapeseed roots became colonized by *P. protegens* CHA0-*gfp2*. Numbers above boxes indicate sample size.

### 10.3. Transstadial transmission of *P. protegens* CHA0, a phenomenon observed in susceptible as well as in resistant insects

Since our experiments with *D. radicum* indicate that the relationship of CHA0 with this insect is rather of commensal than pathogenic nature we wondered to what extent the bacterium can persist in insects highly susceptible to a CHA0 infection. Thus, we monitored the persistence of CHA0 and its effects on insect development in additional insect species: the diamondback moth, *Plutella xylostella*, a leaf-feeder that is highly susceptible to CHA0 [6, 10]; the large white, *Pieris brassicae*, which is another leaf-feeding lepidopteran, for which earlier experiments suggested high susceptibility to CHA0 [7]; and finally the black vine weevil, *Otiorhynchus sulcatus* as a second root feeding insect, which so far had never been investigated for its interaction with CHA0.

***P. xylostella.*** *P. protegens* CHA0 was found in larvae, pupae and adults of *P. xylostella*. Almost 80% of larvae fed on artificial diet inoculated with 10 µl of $OD_{600}$ = 0.1 (high dosage) of CHA0 did not survive until pupation and the rest commonly died in the pupal stage (Fig. 4A, high). Bacterial numbers in larvae and pupae were comparable, while at both developmental stages dead individuals harbored about 100 to 1000 times more bacteria than individuals that were still alive (Fig. 4B). To investigate, whether CHA0 can persist even to the adult stage, we further extracted *P. xylostella* after infection with a ten times lower dosage causing almost no mortality in the larval stage anymore (Fig. 4A, low). In these infections, CHA0 was only detected in six out of fifteen larvae and at very low numbers (Fig. 4C). However, colonization levels increased in the pupal and adult stages (Fig. 4C). Generally, adult emergence was very low, also in control treatments (Fig. 4A), because the *Plutella* feeding assay is optimized for fast killing of larvae upon feeding on CHA0 and not for long term survival of the insects. Nevertheless, the consistent detection of CHA0 in all extracted imagines indicates transstadial transmission in *P. xylostella* from the larval via the pupal to the adult stage.

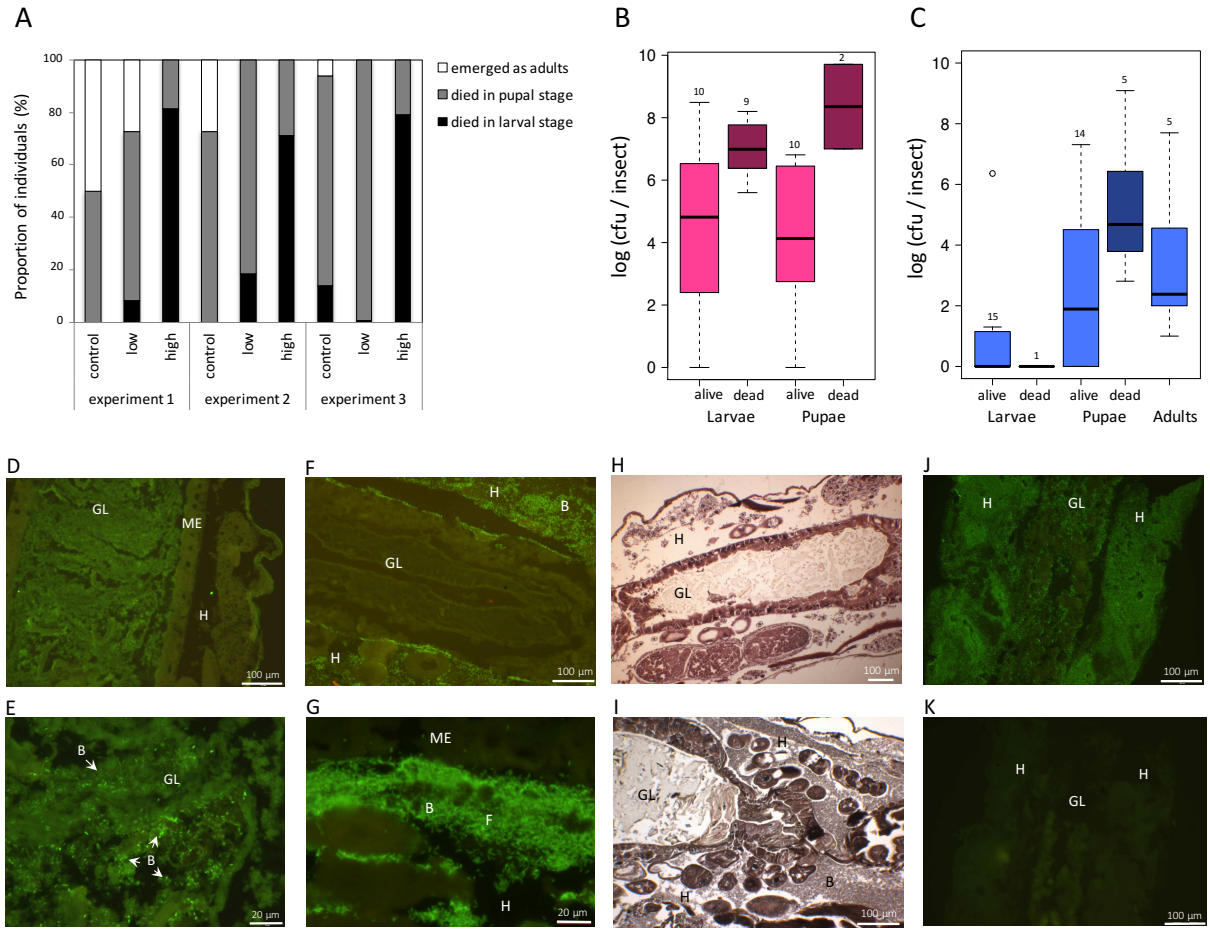


**Figure 4. Infection of Plutella xylostella by *Pseudomonas protegens* CHA0. (A-C)** One-week-old *P. xylostella* larvae (n=32-64) were exposed to artificial diet inoculated with a low dosage (low, 10 µl of $OD_{600}$ = 0.01) or a high dosage (high, 10 µl of $OD_{600}$ = 0.1) of *P. protegens* CHA0-gfp2 or amended with sterile 0.9% NaCl solution (control). **(A)** Fraction of *P. xylostella* larvae dying at larval or pupal stages or emerging as adults. Three repetitions of the experiment are depicted. **(B)** Colonization of *P. xylostella* by *P. protegens* CHA0-gfp2 (high dosage). Data are pooled from experiments 2 and 3. **(C)** Colonization of *P. xylostella* by *P. protegens* CHA0-gfp2 (low dosage). Data are pooled from all three experiments. **(B, C)** Numbers above boxes indicate sample size. No *P. protegens* CHA0-gfp2 was detected in control insects. **(D - K)** Tracking *P. protegens* CHA0 in *P. xylostella* larvae upon oral uptake using microscopy on serial sections of fixed larvae. Larvae were infected with P. protegens CHA1176, a GFP expressing variant of P. protegens CHA0. **(D-G, J)** Sections of these larvae were stained with anti-GFP. The use of anti-GFP antibodies was necessary because fixation of larvae with Duboscq-Brazil's alcoholic Bouin's destroys intrinsic GFP fluorescence. **(H, I)** Sections stained with Heidenhain's iron hematoxylin. **(D, E)** CHA0 in the gut, but not in the hemolymph. **(E)** is a magnification of **(D)**. **(F, G, I)** CHA0 in the hemolymph and in fat body cells, but not in the gut. **(H)** Control larva fed on bacteria-free diet. **(J)** Moribund larva completely colonized by CHA0**. (K)** Consecutive section of **(J)** stained without adding anti- GFP antibody. B, bacteria; F, fat body; GL, gut lumen; H, hemocoel; ME, midgut epithelium.

Although several insect pathogenicity factors of *P. protegens* CHA0 have been identified [16], very little is known about the infection process inside the insect and the damage caused by CHA0. To be able to study in the same insect the localization of CHA0 and histological changes of insect tissues we established a microscopy method on thin sections of *P. xylostella* larvae (Supplementary Methods). Larvae fed on CHA0 containing diet were fixed and consecutive sections were either stained with anti-GFP antibodies or Heidenhain's iron hematoxylin to visualize the bacteria or the insect tissue, respectively. Larvae from two independent experiments were analyzed and representative pictures are shown in Fig. 4 D-K. They give a first insight into the colonization of *P. xylostella* larvae by *P. protegens* CHA0. In most larval samples from early infection time-points CHA0 could not be detected, but in two cases it was found in the midgut lumen (Fig. 4D, E). In contrast, at later stages of infection CHA0 was often found in the hemolymph and the fat body cells while no excessive destruction of the midgut epithelium and no bacteria in the gut could be observed (Fig. 4F, G, I). Finally, moribund larvae were always full of CHA0 all over the hemocoel and the gut and organs were not distinctively recognizable anymore (Fig. 4J).

***P. brassicae.*** In oral infections of *P. brassicae* larvae with *P. protegens* CHA0, survival was found to be dependent on the larval stage. When 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ instar larvae were fed with high dosages of CHA0, approximately 70% to 95.8% of the larvae died within six days (Supplementary Figures S3A, S3B, S3C). In contrast, 4$^{th}$ instar larvae fed with the same number of CHA0 cells were much more resistant and showed survival rates of over 95% in most of the experiments (Supplementary Figures S3D, S4). Therefore, this instar was used to assess persistence of CHA0 throughout different developmental stages (Fig. 5). Still some CHA0-infected 4$^{th}$ instar larvae were unable to form intact pupae (Fig. 5C6) and some pupae with normal

appearance became melanized and died (Fig. 5C7).

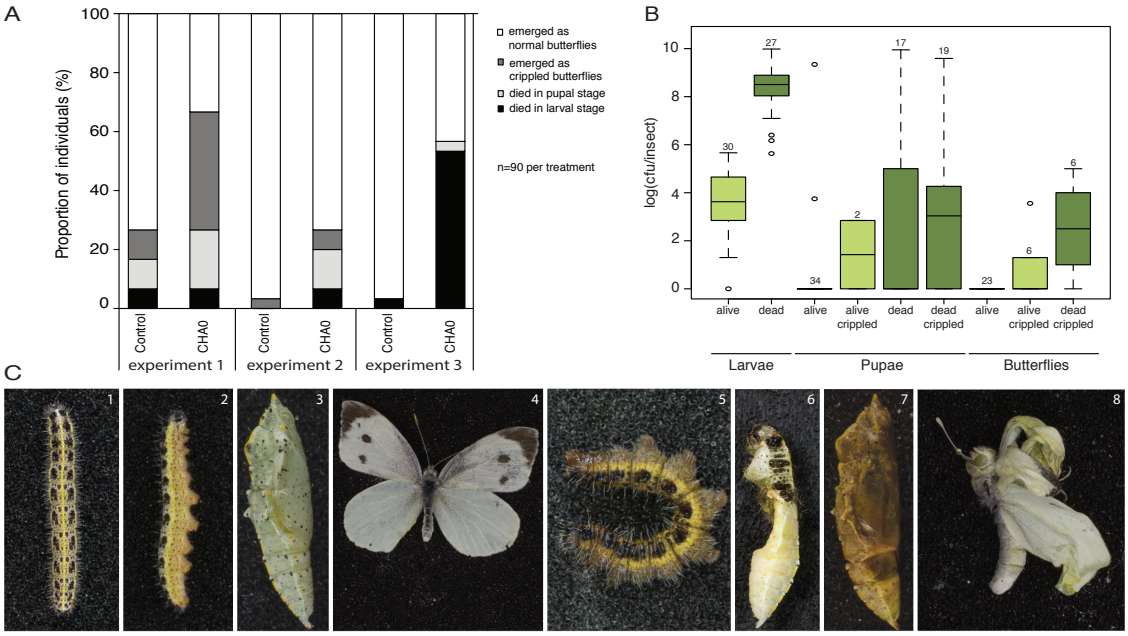


**Figure 5. Infection of *Pieris brassicae* by *Pseudomonas protegens* CHA0. (A-C)** Fourth instar *P. brassicae* larvae were fed on artificial diet inoculated with 10 µl of a *P. protegens* CHA0-gfp2 bacterial suspension ($OD_{600}$= 20) (CHA0) or amended with sterile 0.9% NaCl solution (control). **(A)** Fate of larvae fed with control or CHA0-treated diet. Three repetitions of the experiment are depicted. **(B)** Colonization of *P. brassicae* by *P. protegens* CHA0-gfp2. Data are pooled from three independent experiments. Numbers above boxes indicate sample size. No *P. protegens* CHA0-gfp2 was detected in control insects. **(C)** Phenotypical differences between insect stages developed from control (1-4) and CHA0-treated (5-8) larvae. Healthy larvae (1, 2), pupa (3) and butterfly (4); dead larva (5) and pupa (7); pupa (6) and butterfly (8) with morphological defects.

The number of dead individuals (larvae plus pupae) was higher in the CHA0 treatment compared to the control (Fig. 5A). Moreover, 15.5% of total number of butterflies from the CHA0 treatment emerged with morphological defects, i.e. strongly deformed wings (Fig. 5A, 5C8), which was again higher than in the control (4.4%). Extraction of larvae, pupae and adults revealed presence of CHA0 in nearly all living larvae at levels of around 4 $\log_{10}$ cfu per insect and in dead larvae even at levels as high as 9 $\log_{10}$ cfu per insect while in living pupae and adults with healthy appearance, CHA0 was only found exceptionally (Fig. 5B). In individuals with abnormal phenotypes, CHA0 was often detected, which indicates that in certain cases

the bacterium can survive in *P. brassicae* until the pupal and the adult stages (Fig. 5B).

***O. sulcatus.*** Since data on the interaction of CHA0 with root-feeding insects, beside the *D. radicum* data presented here, is lacking, we performed first small-scale experiments with a second root pest, *O. sulcatus*. CHA0 did not affect the survival of larvae feeding on strawberry roots (Supplementary Figure S5). Still, CHA0 was detected in two thirds (experiment A and B) of the pupae and in two thirds or all (experiment A and B, respectively) of the adults emerging from the CHA0-fed *O. sulcatus* larvae. However, the method used in these experiments only allowed a qualitative detection and no quantification of CHA0 (Supplementary Methods). The results are in line with the data obtained with *D. radicum*. Nevertheless, the observed persistence of CHA0 throughout different life stages of *O. sulcatus* warrants further investigation.

## 5. Discussion

Our results indicate that *D. radicum* is generally less susceptible to *P. protegens* and *P. chlororaphis* than the lepidopteran leaf-feeders tested here and in earlier studies [6, 8, 10]. Moreover, the investigated coleopteran species *O. sulcatus* was not affected at all after feeding on *P. protegens* treated roots. Thus, the pathogenicity of *P. chlororaphis* subgroup bacteria seems to depend on the insect species/order. When comparing susceptibility of different insect species, one has to keep in mind that root and leaf-feeders were tested in different setups. *Plutella* and *Pieris* larvae were kept in small cages and fed on artificial diet or detached leaves, an unnatural environment to the insects and thus potentially stressful. Moreover, bacterial numbers internalized by *D. radicum* feeding on radishes or cauliflower roots were presumably very low, since larvae burrow into the root and *P. protegens*

CHA0 colonizes mainly the root surface [37]. However, in an earlier study by Ruffner et al. [10] PCL1391 and CHA0 were tested against three lepidopteran species in a more natural setting where bacteria were sprayed onto plant leaves. High larval or pupal mortality was obtained for all tested species when leaves were sprayed with a suspension containing $10^6$ CFU/ml resulting in an uptake of probably only $10^4$ - $10^5$ cells, which indicates that Lepidoptera are indeed highly susceptible to these bacteria.

In our *Delia* experiments, we observed differences between *Pseudomonas* strains, which is in line with earlier studies [6, 8] where insecticidal activity also had a strain specific component. While *P. protegens* PF had no negative effects on *D. radicum* larvae, the reduction of larval survival and pupal size in the treatment with PCL1391 and CMR12a suggests that these strains can be mildly pathogenic to *D. radicum*. For biocontrol purposes, bacterial effects might be increased by exposing *D. radicum* to additional stress, e.g. by combining bacteria with organisms that could facilitate the access to the hemocoel, such as entomopathogenic fungi or nematodes.

*P. protegens* CHA0 did not affect survival of *D. radicum* larvae, but it was able to persist in the insect throughout different life-stages. Thus, CHA0 seems to follow different ecological strategies depending on the insect host. It can be highly pathogenic, for instance towards *P. xylostella* and *P. brassicae*, but, as our results with *D. radicum* and *O. sulcatus* indicate, it can also live in insects as a commensal or, possibly, an opportunistic pathogen. Moreover, this study provides first evidence that in such a commensal association CHA0 could be transmitted by the insect from the roots of one plant to the roots of another plant. Hence insects might not only serve as additional hosts for *P. chlororaphis* subgroup bacteria, but also as vectors. In the following, we will discuss the interaction of CHA0 with non-susceptible and susceptible insect hosts.

## 10.1. Interaction with non/little susceptible insect hosts

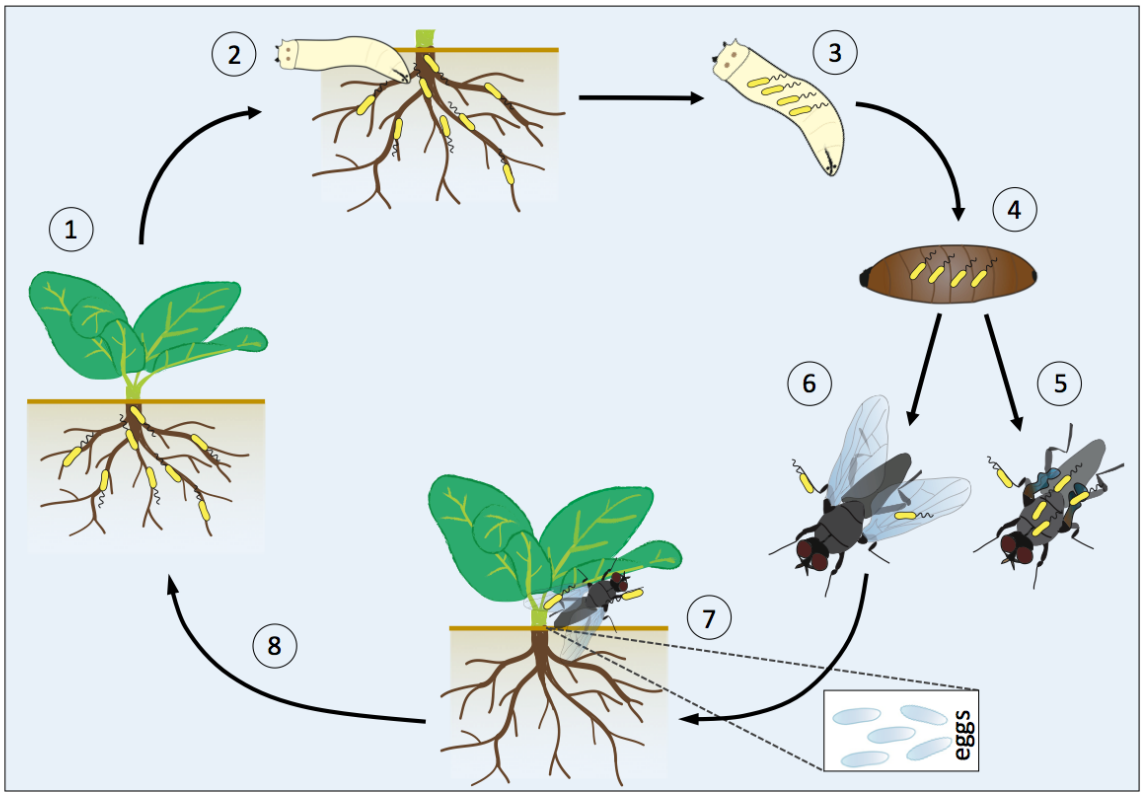


**Figure 6. *Pseudomonas protegens* CHA0, taken up by root-feeding *Delia radicum* larvae, persists in the insect throughout different life stages and can be dispersed to new host plants.** Roots of cauliflower plants are colonized by *P. protegens* CHA0 **(1)** and freshly hatched larvae of *D. radicum* are feeding on them **(2)**. CHA0 becomes ingested by the larvae **(3)** and persists inside the insect also in the pupal **(4)** and adult stage **(5, 6)** (Figure 2B). Emerging flies occasionally exhibit morphological defects **(5),** which affects mainly the wings (Figure 2C). In nature, crippled flies are not able to reproduce, which will cause a decline of the insect population. However, healthy flies **(6)** will mate and females will search for a new host plant where they deposit their eggs in the immediate vicinity of the stem **(7)**. Flies are able to transmit CHA0 to a new host plant resulting in bacterial colonization of the roots **(8)** (Fig. 3).

A summary of a potential association of *P. protegens* CHA0 with *D. radicum* throughout the insect life-cycle is depicted in Fig. 6. *P. protegens* becomes internalized by root feeding *Delia* larvae, persists until the pupal stage and emerging flies can transmit the bacterium to the roots of a new host plant (Fig. 6). This could, for instance, occur when female flies lay eggs next to plant stems thereby delivering bacteria directly into a new soil habitat. In the following, the bacterium colonizes the roots of the new host plant and can again colonize larvae that hatched from deposited

eggs (Fig. 6).

The results obtained with the second root feeder investigated, *O. sulcatus*, suggest that the persistence of CHA0 throughout insect development in non-lethal interactions is not restricted to *Delia*, but might be a general trait of this bacterium. Transstadial transmission (from larva to adult) of bacteria that do not have an intracellular lifestyle, which is common for endosymbionts [38, 39], is reported for several insect species [40–43]. However, in other cases bacteria are lost during the pupal stage, before adult emergence [41–44]. Our qualitative approach, where entire flies were incubated in selective medium, detected CHA0 associated with adult *D. radicum* in contrast to our extraction method (detection limit of the extraction method: 50 cfu/ fly). Moreover, our transfer experiments showed that the bacterium was transmitted by *Delia* flies in about 90% of the cases. These results indicate that emerging flies regularly carry CHA0 but in very low numbers. Due to repeated molting and metamorphosis, the insect represents an unstable habitat for microbes, though bacteria might still persist in specialized crypts or paunches present in the guts of many insect species [38]. Persistence of CHA0 in insects of different orders each of which harbors specific anatomical and developmental features might rely on different strategies. Although CHA0 did not reduce *Delia* survival, we occasionally observed an effect on insect development, i.e. reduced pupal size and morphological defects in adults. The latter was also found for the susceptible species *P. brassicae* and similar observations are reported for *D. melanogaster* larvae infected with the related strain *P. protegens* Pf-5 [11, 18] and for leaffolder moths (*Cnaphalocrocis medinalis*) fed with rice leaves treated with a mix of *P. fluorescens* strains [45].

As vectors, insects would allow the bacteria to overcome large distances and to conquer new root habitats, which might considerably influence the spread of root-colonizing fluorescent pseudomonads. Insect-mediated dispersal is also known

for several plant- pathogenic bacteria, but most of them do not exhibit insecticidal activity [22]; for instance *Xylella fastidiosa*, causing citrus variegated chlorosis and Pierce's disease of grape, can be transmitted from one plant host to another by sharpshooter leafhoppers and spittlebugs [46]. Still, in some associations plant-pathogens are also insect pathogenic. *Pseudomonas syringae* B728a can cause high mortality rates in the pea aphid and at the same time, it is excreted with honeydew and can thereby be dispersed by moving aphids [47]. Experiments carried out with sterile transmission microcosms in our study provide only a first proof of principle for the possibility of insect-mediated dispersal. In nature, a few arriving CHA0 cells might encounter considerably higher difficulties to establish on the roots of a new plant host due to competition, predation or unfavorable environmental conditions. Therefore, transmission of CHA0 by insects might be a rare event under natural conditions and difficult to investigate experimentally. Nevertheless, in an experiment in which we added increasing concentrations of CHA0 cells to rapeseed seedlings grown in natural soil, we found root colonization by CHA0 to occur in 4% and 55% of the pots upon addition of as few as 50 or 500 cells, respectively (our unpublished results). This indicates that already a few transmitted CHA0 cells might be able to establish on the roots of a plant even when facing competition with a natural soil microbiome.

### 10.2. Interaction with susceptible insect hosts

We wondered whether CHA0 can also persist until the pupal and adult stages in insects in which the bacterium can cause lethal infections. Larvae of *P. xylostella* and *P. brassicae* are susceptible to CHA0 and become highly colonized. In *P. xylostella,* CHA0 generally persists in larvae and pupae but the insects seem to succumb to the infection sooner or later. However, when low dosages were fed and

some individuals survived until the adult stage, we could detect CHA0 in these adults (Fig. 4C). In contrast, a considerable fraction of 4$^{th}$ instar *P. brassicae* larvae was able to eradicate CHA0 before or during pupation. However, those *Pieris* larvae, in which CHA0 was able to persist, seemed to succumb to the infection during pupation or exhibited major developmental defects (Fig. 5B, 5C5-8). Taken together, CHA0, when ingested by larvae was able to persist until the adult stage in four investigated insect species irrespective of their susceptibility pointing to the dispersal by adult insects as a possible scenario.

Finally, we wanted to take a closer look at the course of infection in a susceptible insect. During a lethal infection, *P. protegens* CHA0 multiplies to very high numbers as shown in Fig. 4 and reported earlier [6, 7, 10, 19]. Extraction of entire larvae does not allow any conclusion on the localization of the bacteria. The here presented microscopy method enabled the visualization of *P. protegens* CHA0 during the insect infection and, in parallel, the observation of histopathological changes in *P. xylostella* larvae. Observations indicate that *P. protegens* CHA0 does not colonize the gut to very high numbers and does not cause complete rupture of the midgut epithelium. Therefore, we hypothesize that *P. protegens* CHA0 colonizes a restricted area of the gut where it is able to enter the hemocoel. Once in the hemocoel the bacteria multiply exponentially causing a fatal septicemia. The use of the insect body as a nutrient source and a mass replication vessel is supported by the pictures of moribund larvae as well as by the very high bacteria counts in dead individuals of *P. xylostella* and *P. brassicae*. The pictures presented here give only a first insight into the colonization and infection process. How and where exactly the bacterium overcomes the gut barrier in order to invade the hemolymph remains to be discovered and requires in-depth microscopy studies.

## 6. Conclusions

This study investigated for the first time how *P chlororaphis* subgroup bacteria, known for their insecticidal activity against leaf-feeding insects, interact with a root-feeding insect present in their rhizosphere habitat. While the reduction of larval survival by certain strains gives hope for potential applications as biocontrol organisms of root pests. Such as *D. radicum*, the discovery of persistence of *P. protegens* CHA0 in insect through different developmental stages and its dispersal to a new host plant adds novel and intriguing aspects to the ecology of plant-colonizing pseudomonads. Besides being plant-beneficial rhizosphere inhabitants, they can also live in insects as pathogens or commensals. Indeed, in a recent survey we could detect *P. chlororaphis* and *P. protegens* in about 10% of sampled soil arthropods (our unpublished results). Thus, these bacteria seem to be much more versatile than previously thought and we still far from fully understanding the ecology. It remains subject to future research to discover additional habitats these bacteria might have conquered and to elucidate how they manage to switch between very different, e.g., root- and insect-associated life-styles.

## 7. Conflict of Interest

The authors declare no conflict of interest

## 8. Acknowledgments

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feeding assay. We also thank Ethan Stewart for his help with the measurement of the size of *D. radicum* pupae by an ImageJ macro. We thank Helga Radke for technical assistance on preparation of insect samples for microscopy and Alexander Rapp from Technical University Darmstadt as well as Astrid Zimmermann for providing anti-GFP antibodies and help on the immunofluorescence microscopy. Furthermore, we acknowledge Johannes Jehle for his support to develop the collaboration between ETH Zurich and JKI Darmstadt. Finally, we thank Dylan Bär, Jana Schneider, Sandra Siegfried, Maria Haller and Anja Taddei for technical assistance and Tobias Löser and Maria Péchy-Tarr for help with the generation of GFP-tagged strains.

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# 10. Supplementary Information

## 10.1. Supplementary Methods

### Persistence/transmission of *Pseudomonas protegens* CHA0 in experiments with cauliflower/rapeseed and *Delia radicum*

*Growing and inoculation of cauliflower plants*

Cauliflower seeds (*Brassica oleracea botrytis* 'Walcheren Winter 5', Samen Mauser AG, Switzerland) were surface-sterilized as follows: seeds were placed for 2 min in 70% ethanol, thoroughly washed with sterile dd$H_2O$, subsequently placed for 30 min in 4% NaOCl, and again thoroughly washed with sterile dd$H_2O$. Surface-sterilized seeds were pre-germinated for six days on 1% water agar at 24°C in the dark. Individual seedlings were transferred to pots (one seedling per pot) of which the lower two thirds were filled with autoclaved potting soil (TREF Go PP 7000 plant substrate, GVZ Rossat AG, Switzerland) and the upper third was filled with a mix of different fractions of quartz sand and vermiculite [1]. Each pot was amended with 10 ml of bacterial suspension (CHA0-*gfp2*, $OD_{600}$ = 0.45; an $OD_{600}$ of 0.125 contains about $10^8$ cfu/ml) or water (control) and four trays, each containing twelve pots, were prepared for each treatment. Cauliflower plants were then grown for three weeks in a growth chamber with a 16-h day (20°C, 210 µmol $m^{-2}$ $s^{-1}$), 8-h night cycle (18°C) and a relative humidity of 80%. For application of *Delia radicum* to cauliflower plants, a small piece of blue paper containing five freshly hatched larvae was placed next to the stem of the plants. Larvae that did not manage to enter the soil were replaced to ensure equal numbers of viable larvae on the roots.

*Assessment of root colonization by Pseudomonas protegens CHA0-gfp2*

Root colonization was assessed in four pots per tray of the *P. protegens* CHA0-*gfp2* treated plants and in all control pots to ensure that these were not contaminated with CHA0. Roots of cauliflower plants were washed on a sieve to remove adhering substrate.

Then subsamples of the roots were placed in Eppendorf tubes containing 0.9% NaCl solution and incubated at 3°C over-night. Next, samples were shaken for 30 min at 1400 rpm on an Eppendorf thermomixer compact at 4°C. Serial dilutions were plated onto King's B (KB) agar plates [2] supplemented with chloramphenicol (13 μg $ml^{-1}$), cycloheximide (100 μg $ml^{-1}$), ampicillin (40 μg $ml^{-1}$) and gentamicin (10 μg $ml^{-1}$) and plates were incubated at 27°C for two days. Colony forming units (cfu) were checked for expression of GFP with a Leica DM2500 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). In a few pots of the control treatment, a contamination by *P. protegens* CHA0-*gfp2* was observed. These samples were excluded from the analysis and emerging pupae not used for further experiments.

*Transmission experiment with Delia radicum*

Rapeseed seeds were sterilized and pre-germinated as described above for cauliflower seeds with the only difference that pre-germination lasted only one day instead of six days. Plastic beakers (500 cc) with a lid (Riwisa AG, Switzerland) were partly filled with 3 cm of autoclaved sand-vermiculite mix supplemented with 35 ml of Knop plant nutrient solution [1]. Four pre-germinated seeds were planted per beaker and grown for 18 days in a growth chamber with a 16-h day (20°C, 210 μmol $m^{-2}$ $s^{-1}$), 8-h night cycle (18°C) and a relative humidity of 80%. Then, another 10 ml of Knop solution was added as well as sterile lids of Eppendorf tubes, one containing wet fly diet and one containing dry fly diet [3]. Three to four pupae that had emerged from control or *P. protegens* CHA0-*gfp2* treatments in the cauliflower experiment (see above) were added per beaker in small sterile Erlenmeyer flasks (experiment 1) or sterile lids of Eppendorf tubes (experiment 2). In experiment 1, three of these transmission microcosms were established for the control treatment and fourteen for the CHA0-*gfp2* treatment. Of the latter one sample was excluded from the analysis, because no flies hatched. In experiment 2, nine transmission

microcosms per treatment were established. Flies started to hatch after one day. Nine days later roots of rapeseed plants were checked for colonization by *P. protegens* CHA0-*gfp2* as described above for cauliflower plants. Roots of plants grown in the same beaker were pooled for analysis.

**Experiments with *Otiorhynchus sulcatus***

Larvae of *O. sulcatus* and strawberry plants were kindly provided by Matthias Lutz (ZHAW Wädenswil, Switzerland). Root balls of two months old strawberry plants ('Elsanta', Näppbrunnenhof, Switzerland) were incubated for 10 min in a cell suspension of *P. protegens* CHA0 ($OD_{600}$ = 0.0125) or in sterile water for control treatments and in the following planted in pots containing potting soil (TREF Go PP 7000 plant substrate, GVZ Rossat AG, Switzerland). Fifteen (experiment A) or ten (experiment B) last-instar larvae of *O. sulcatus* were added per pot. Plants were kept at 18°C day temperature, 15°C night temperature, 60% humidity and a 16-h day, 8-h night cycle in a growth chamber. After one month, pupae were harvested and either directly extracted or maintained until adults emerged. Three pupae and three adults of both treatments were surface sterilized (30 s 70% ethanol, rinsed in 0.9% NaCl solution) and homogenized in sterile 0.9% NaCl solution with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). The resulting suspensions were serially diluted and plated onto KB agar plates supplemented with chloramphenicol (13 μg $ml^{-1}$), cycloheximide (100 μg $ml^{-1}$) and ampicillin (40 μg $ml^{-1}$). Since for these experiments *P. protegens* CHA0 without a GFP-tag was used, the identity of a subsample of the growing colonies was checked as described by Ruffner [4] with a colony PCR using primers that specifically amplify *P. protegens* CHA0 [5] and by sequencing a part of the *16s rRNA* gene. This method allowed only a qualitative detection and no quantification of CHA0. The experiment was conducted twice.

**Survival and colonization experiments with *Plutella xylostella***

The experiments with *Plutella xylostella* were conducted as detailed in Flury et al. [6]. One-week-old *P. xylostella* larvae were kept each separately in multi-well plates and exposed to artificial diet inoculated with 10 µl of bacterial suspension of an $OD_{600}$ of 0.1 (high dosage) or 0.01 (low dosage). Experiments 1 and 2 were set up with 32 larvae per treatment, experiment 3 with 64 larvae per treatment. Larvae and pupae were considered dead when they did not react to poking. From each treatment, five individuals per developmental stage (alive and dead) or as many as available were extracted as described under 'Assessment of bacterial colonization rates' in the main paper.

**Efficacy of insect surface sterilization**

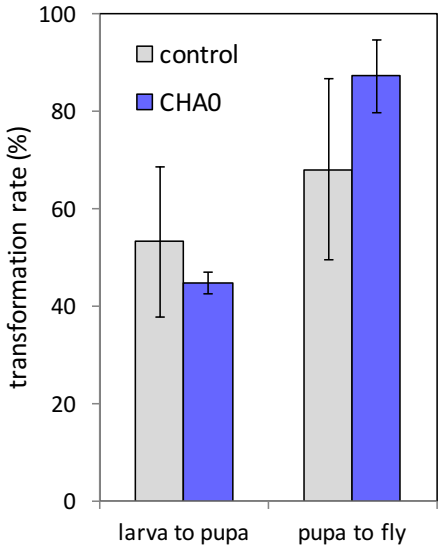
The efficacy of insect surface sterilization was evaluated the following way: 500 CHA0 cells were added to pupae and larvae. Half of the insects were surface sterilized and half left untreated as control. Next, insects were placed in Eppendorf tubes containing sterile 0.9% NaCl solution, tubes were agitated and the resulting suspension was plated onto KB agar. The same test was performed with insects derived from the experiments (in vitro-feeding assays and pot assays). In addition, some surface sterilized and non-surface sterilized pupae were carefully rolled over KB agar. For pupae, the surface sterilization was highly effective and we never detected any CHA0 colonies on KB agar. On surface-sterilized larvae, we detected CHA0 in some cases, but only 5-10% of the cell numbers compared to the non-surface sterilized control. However, it is not possible to tell, whether the detected cells really derived from the insect surface or whether they were released by the insects during the shaking process.

**Microscopy**

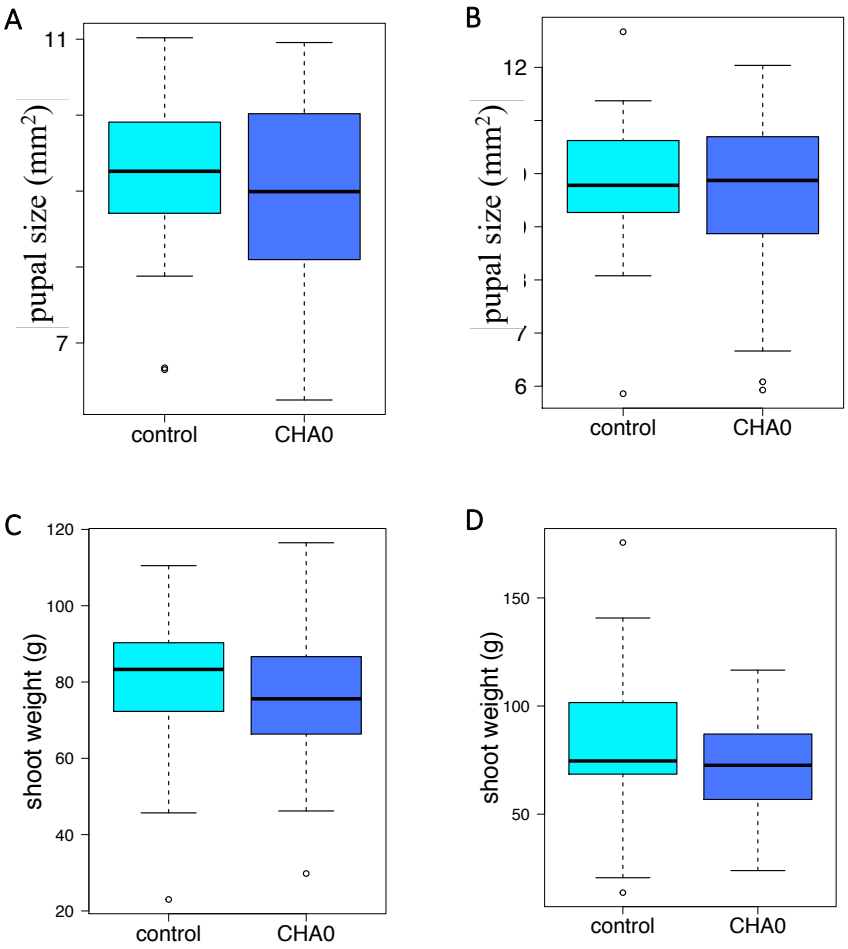
For microscopy, twelve one-week-old *P. xylostella* larvae were kept together in a Petri

dish which was lined with a wetted filter paper and contained four pellets of artificial diet. For the bacterial treatments diet was inoculated with 10 µl of bacterial suspension of $OD_{600}$ = 10, which corresponds to about 8 x $10^7$ cells. Larvae were collected at different time points after infection (a total of 27 infected larvae were investigated by microscopy), were killed by exposure to ethyl acetate, and subsequently fixed for 24 h in Duboscq-Brazil's alcoholic Bouin's (saturated alcoholic solution of picric acid, formaldehyde, glacial acetic acid, 10:4:1 [vol/vol]). After dehydration in ascending concentrations of ethanol, larvae were embedded in Histosec (Merck, Darmstadt, Germany). Embedded larvae were cut into serial sections of 6 µm, mounted onto microscope slides and cleared from Histosec with xylene. For histopathology analysis, sections were stained with Heidenhain's iron hematoxylin, counterstained in erythrosine and examined in a Leica photomicroscope, model DMRB (Leica, Wetzlar, Germany). To be able to identify the applied bacteria, the GFP tagged variant *P. protegens* CHA1176 (Table 1) was used instead of wild type CHA0. However, fixation in Duboscq-Brazil's alcoholic Bouin's destroys intrinsic GFP fluorescence and immunofluorescence microscopy was needed to specifically detect the bacteria. To allow access of the antibodies to the intracellular GFP, tissue sections were boiled for 30 min at 90°C in 10 mM sodium citrate, washed in PBS and blocked in 1% BSA, 0.3%Triton X-100 in PBS as described by Benjamin et al. [7]. Sections were then incubated in monoclonal mouse anti-GFP IgG (1:500, Roche, Switzerland) for 1 h at room temperature and subsequently over-night at 4°C. After washing three times in PBS, sections were incubated with donkey anti-mouse IgG-FITC (1:200, Dianova, Germany) for 4 h at room temperature and were examined with a Leitz Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany). All images were captured with an SIS ColorView II camera (Soft Imaging System GmbH, Münster, Germany).

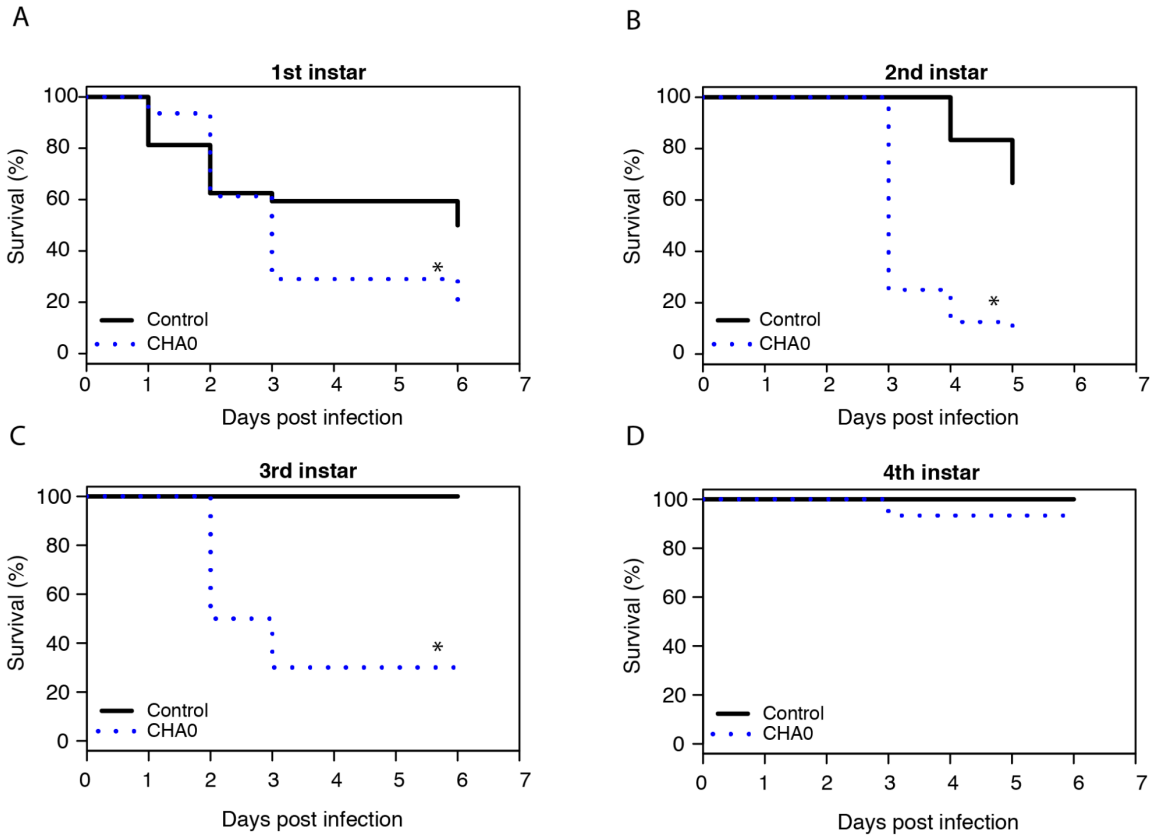
## 10.2. Supplementary figures



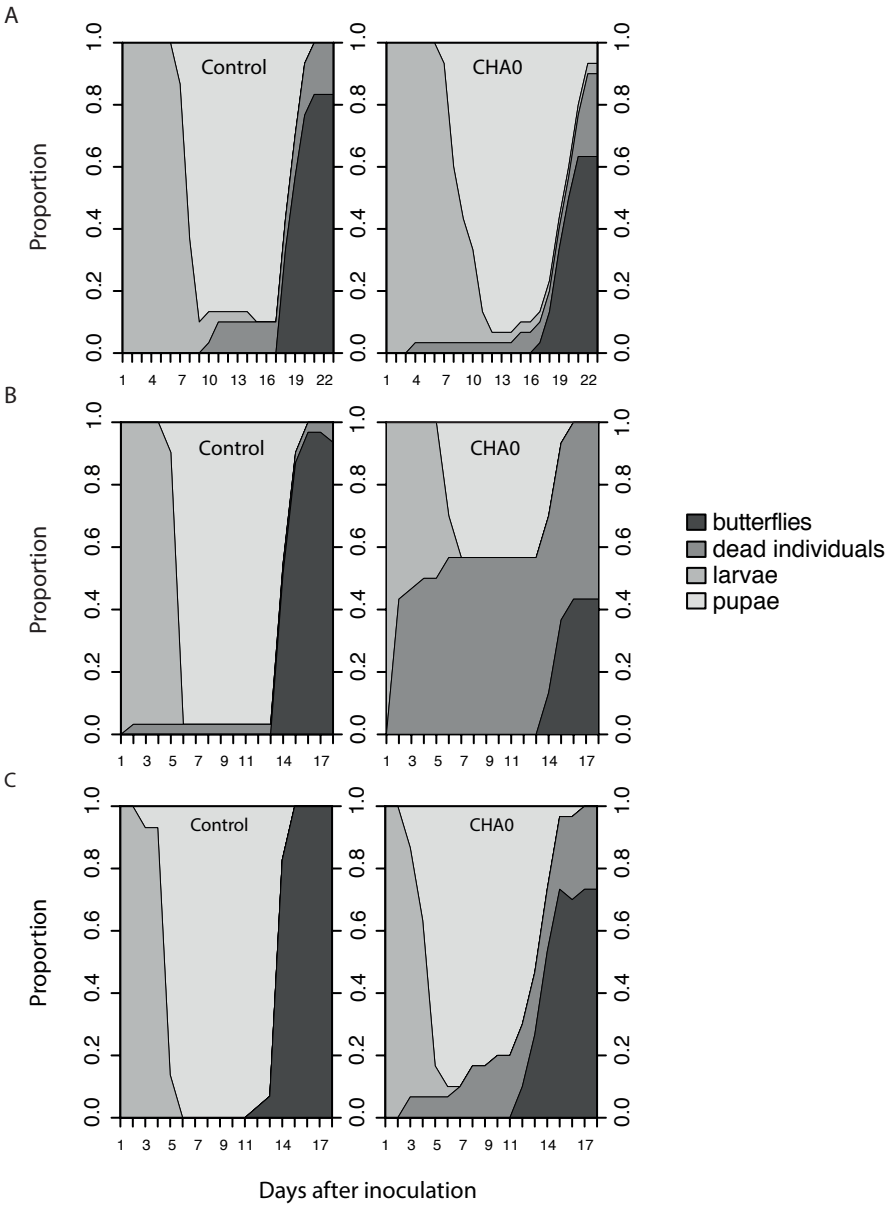

**Supplementary Figure S1. *Pseudomonas protegens* CHA0-*gfp2* does not affect survival of *Delia radicum*.** This experiment is a repetition of the one depicted in Figure 2A. Five freshly hatched *D. radicum* larvae were added to cauliflower plants (four trays per treatment, each containing twelve pots) grown with *P. protegens* CHA0-*gfp2* (CHA0) on the roots or without (control). Pupation rate and the rate of flies emerging from pupae did not significantly differ between the control and the *P. protegens* CHA0-*gfp2* treatment ($p < 0.05$; Student's t test). Error bars depict standard deviations of the mean of four replicate trays.



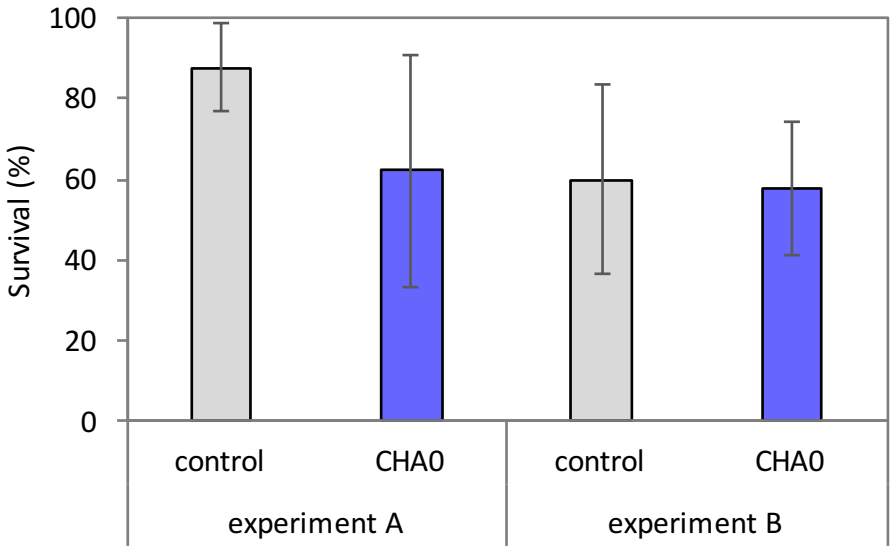

**Supplementary Figure S2. *Pseudomonas protegens* CHA0-*gfp2* does neither reduce pupal size of *Delia radicum* nor increase the shoot weight of cauliflower plants infested with the insect.** Roots of cauliflower plants were inoculated with a cell suspension of *P. protegens* CHA0-*gfp2* (CHA0) or amended with water (control) at planting (n = 48). Three weeks later, five freshly hatched *D. radicum* larvae were added and let feed on the roots until pupation. Then the size of emerged pupae **(A, B)** as well as the shoot weight of cauliflower plants **(C, D)** was assessed. For both parameters, no significant difference between the control and the CHA0 treatment could be detected ($p < 0.05$; Mann-Whitney *U* Test) in both repetitions of the experiment **(A, C)** and **(B, D)**. Results of experiment 1 and 2 are depicted in **(A, C)** and **(B, D)**, respectively.




**Supplementary Figure S3. First, second and third instar *Pieris brassicae* larvae, but not fourth instar larvae are highly susceptible to an infection with *Pseudomonas protegens* CHA0.** Kaplan-Meier survival graphs of different larval stages of *P. brassicae* treated with *P. protegens* CHA0. First **(A)**, second **(B)**, third **(C)** and fourth **(D)** instar larvae (n=24 to 32) of *P. brassicae* were fed with a pellet of artificial diet inoculated with ~$10^8$ bacteria or amended with 0.9% NaCl solution (Control). Only larvae that consumed the entire piece of diet were included in the analysis. Asterisks indicate significant differences according to a Log-Rank test ($p \leq 0.05$, Survival Package in R).




**Supplementary Figure S4: Impact of *Pseudomonas protegens* CHA0-*gfp2* on development and mortality of *Pieris brassicae* over time**. Thirty-two fourth-instar *P. brassicae* larvae were fed with artificial diet inoculated with 10 $\mu$l of a bacterial suspension of $OD_{600}$=20 (an $OD_{600}$ of 0.125 corresponds to about $10^8$ cfu/ml) or amended with 0.9% NaCl solution (control). The different development stages of the insects were monitored until the butterflies emerged from the pupae. The experiment was conducted three times (A, B and C).




**Supplementary Figure S5. *Pseudomonas protegens* CHA0 does not affect survival of *Otiorhynchus sulcatus* larvae.** The roots of strawberry plants (one plant per pot, six and five pots per treatment in experiment A and B, respectively) were either inoculated with *P. protegens* CHA0 (CHA0) or mock (control). Fifteen (experiment A) or ten (experiment B) *O. sulcatus* larvae were added per pot. After one month insect survival per pot did not significantly differ between the control and the CHA0 treatment in both experiments ($p < 0.05$; Student's t test). Error bars depict standard deviations of the mean.

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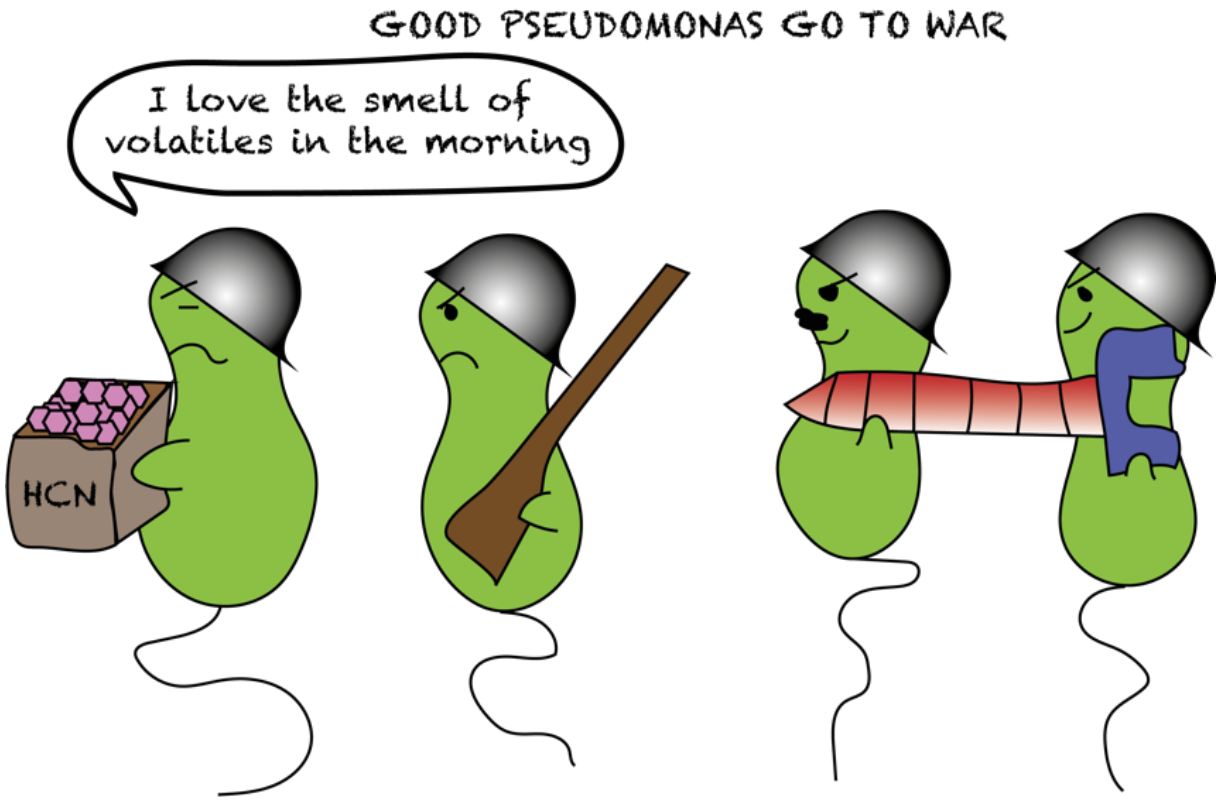
# Chapter 3

## Transcriptome plasticity underlying plant root colonization and insect invasion by *Pseudomonas protegens*



**This chapter was submitted for publication in The ISME journal (2020) by,**

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# 1. Abstract

*Pseudomonas protegens* shows high degrees of lifestyle plasticity since it can establish both plant-beneficial and insect-pathogenic interactions. While *P. protegens* protects plants against soilborne pathogens, it can also invade insects when orally ingested leading to the death of susceptible pest insects. The mechanism whereby pseudomonads effectively switch between lifestyles, plant-beneficial or insecticidal, and the specific factors enabling plant or insect colonization are poorly understood. We generated a large-scale transcriptomics dataset of the model *P. protegens* strain CHA0 which include data from the colonization of wheat roots, the gut of *Plutella xylostella* after oral uptake and the *Galleria mellonella* hemolymph after injection. We identified extensive plasticity in transcriptomic profiles depending on the environment and specific factors associated to different hosts or different stages of insect infection. Specifically, motor-activity and Reb toxin-related genes were highly expressed on roots but showed low expression within insects, while certain antimicrobial compounds (pyoluteorin), exoenzymes (a chitinase and a polyphosphate kinase) and a transposase exhibited insect-specific expression. We further identified two-partner secretion systems as novel factors contributing to pest insect invasion. Finally, we use genus-wide comparative genomics to retrace the evolutionary origins of cross-kingdom colonization.

## 2. Introduction

*Pseudomonas* is a highly versatile genus that comprises bacteria living in diverse environments and that colonize an ecologically-broad range of hosts [1–3]. Some pseudomonads are pathogens of plants or animals such as fish, insects or mammals [3, 4]. Members of the *Pseudomonas fluorescens* group [1, 2] are known plant growth-promoting rhizobacteria (PGPR) that stimulate plant growth, induce systemic resistance against foliar diseases and control soilborne fungal pathogens [5–8]. Due to these characteristics, several *Pseudomonas*-based biocontrol products are currently deployed to control fungal and bacterial diseases [9, 10]. Microbial-based methods for pest control will be crucial in future agricultural practices because an increasing number of chemical fungicides and insecticides is already or will likely be banned due to environmental and health concerns [11–13]. Within the *P. fluorescens* group, the two species *Pseudomonas protegens* and *Pseudomonas chlororaphis* are particularly interesting for plant protection applications because, unlike other biocontrol pseudomonads, they are crop plant colonizers with antifungal activity and pest insect colonizers with insecticidal activity [14–16].

*P. protegens* and *P. chlororaphis* colonize the insect gut after oral intake and transmigrate into the hemolymph, causing systemic infections and the eventual death of several Lepidoptera, Diptera, Coleoptera, and Hemiptera insect species [15–23]. The *P. fluorescens* subgroup [2] harbours insecticidal strains with lower pathogenicity than the *P. protegens*/*P. chlororaphis* species [14, 16, 18, 22]. In addition, *Pseudomonas aeruginosa* and *Pseudomonas entomophila* are also able to infect and kill different insect species, through different mechanisms [24, 25].

The first insecticidal trait discovered was the Fit toxin [17] typically produced by strains belonging to the *P. protegens* and *P. chlororaphis* species [16]. The contribution of this protein toxin to oral and systemic insecticidal activity and its tight insect host-dependent regulation

were studied in some detail in *P. protegens* type strain CHA0 [15, 20, 26, 27]. Fit toxin production only partially explains the insecticidal capabilities of these bacteria as *fit* deletion mutants retain some toxicity [15, 20]. Studies on *P. protegens* CHA0 and other *P. protegens*/*P. chlororaphis* strains related insecticidal activity and host persistence to additional factors, including type VI secretion components [28], chitinase and phospholipase C [16], hydrogen cyanide [29], the cyclic lipopeptide orfamide [29, 30], the toxins rhizoxin [31] and IPD072Aa [32], and specific lipopolysaccharide O-antigens [33]. *P. protegens*/*P. chlororaphis* strains can also cause non-lethal infections [18, 22, 23, 31]. Even if the infection does not kill the insect after oral uptake, strains such as CHA0 can persist until pupal and imago stages, thus affecting the insect development as shown for *Delia radicum*, *Plutella. xylostella* and *Pieris brassicae*, and be transmitted to new host plants by *D. radicum* [23]. Therefore, the ability of *P. protegens* to colonize cross-kingdom insect and plant hosts is impressively demonstrated by work on the model strain CHA0. However, it remains largely unknown what specific traits underlie cross-kingdom host colonization and how plastic responses including transcriptional remodelling contribute to switching between hosts.

We analyzed the transcriptome of *P. protegens* CHA0 during the colonization of plant roots, as well as from different compartments of insect hosts, specifically the hemolymph and gut, representing different stages of infection. We provide the first evidence for transcriptome remodelling underlying switches between insect pathogenic and plant beneficial lifestyles. We showed that CHA0 uses a host-specific set of tools for roots and for different insect compartments. Finally, we use genus-wide comparative genomics to retrace the evolutionary origins of cross-kingdom host colonization.

# 3. Material and Methods

## 11.1. Preparation of *P. protegens* CHA0 samples from different hosts and environments

For each host/environment four independent replicate samples were prepared. From all samples an aliquot was used for assessment of bacterial numbers by plating serial dilutions onto King's B+++ agar (see Supplementary Methods) [34, 35]. The remaining samples were immediately frozen in liquid nitrogen.

*Grace's insect medium and lysogeny broth. P. protegens* CHA0 was grown on KB+++ agar for two days. Single colonies were transferred to lysogeny broth (LB) [36] or Grace's insect medium (GIM. Sigma Aldrich, MO, USA) and grown to $OD_{600}$ = 1.74 – 1.86 (~1.5 x$10^9$ cells/ml) at 24 °C while shaking at 180 rpm. Four ml of cultures were centrifuged (7000 rpm) and pellets used for RNA extractions.

*Wheat roots*. Root colonization assays were performed as described in de Werra et al., [37] and further explained in Supplementary Methods. Briefly, pre-germinated seeds of spring wheat, variety Rubli, were inoculated with 1 ml of a suspension containing $10^8$ CHA0 cells/ml and placed into seed germination pouches. Plants were grown at 22 °C and 60% humidity with a 16/8 h day (270 µmol $m^{-2}s^{-1}$)/night cycle. After one week, roots of 99 plants per replicate were harvested, shaken in 0.9% NaCl, the resulting suspensions were centrifuged and pellets containing bacteria were pooled for RNA extraction.

*P. xylostella gut (oral infection). P. xylostella* eggs were kept before and during the experiment at 25 °C, 60% relative humidity and a 16/8 h day/night cycle with 162 µmol $m^{-2}s^{-1}$. Second instar *P. xylostella* were fed with pellets of artificial diet spiked with 4 x $10^6$ CHA0 cells or NaCl 0.9% (control) as previously described by Flury et al., [29] and further explained in Supplementary Methods. For each replicate 120 treated alive larvae were collected 24 or 36 h post-feeding, surface disinfected and homogenized and homogenates were pooled. Sixty-three to sixty-five larvae per treatment were used for assessing survival over time.

*G. mellonella hemolymph (hemocoel injection).* Seventh instar *G. mellonella* larvae were injected with 2 x $10^3$ CHA0 cells or 0.9% NaCl solution as previously described by Flury et al., [29] and further explained in Supplementary Methods. After 24 h, 55 alive non-melanized larvae per replicate were surface disinfected, one leg was cut and the hemolymph collected. Thirty to fifty larvae per treatment were used to assess survival.

### 11.2. RNA extraction and sequencing

The range of total numbers of CHA0 cells used for sequencing were: LB, 5.6 – 5.9 x $10^9$; GIM, 5.5 – 5.9 x $10^9$; wheat, 5 x $10^8$ – 2 x $10^{10}$; *P. xylostella* 24h, 2.8 x $10^6$ – 4.1 x $10^7$; *P. xylostella* 36h, 2.4 x $10^6$ – 1.6 x $10^8$; and *G. mellonella,* 8.2 x $10^7$ – 2.2 x $10^9$ cells. RNA from insect and media samples was extracted using the GENEzol Reagent (Geneaid International, Taiwan) and from wheat root samples using the RNAeasy Plant minikit™ (Qiagen, Germany) without bead-ruption. All extracts were treated with DNase from the RNeasy mini kit™ (Qiagen, Germany). RNA quality was assessed using the 2200 Tapestation (Agilent, CA, USA) and Nanodrop 2000 (Thermo Scientific, MA, USA). For details, see Supplementary Table S1. Libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit. The RiboZero Bacteria kit was used for medium samples, RiboZero Epidemiology for insect samples and Ribozero Plant Root for wheat samples to remove bacterial, insect and plant rRNA, respectively (Illumina, CA, USA). Samples were sequenced using Illumina NextSeq500 single-end 81 bp sequencing (Illumina, CA, USA) at the Genomics Facility BSSE in Basel, Switzerland.

### 11.3. Bioinformatics analysis

*Read trimming, alignment and normalization*. Raw reads were quality trimmed and filtered for adaptor contamination with Trimmomatic 0.36 [38]. Processed reads were aligned against

the *P. protegens* CHA0 genome (NCBI entry number LS999205.1, [39]) using STAR 2.3.5a [40]. Mapped reads were counted with featurecounts 1.5.3 [41] using the stranded option and normalized with the TMM (trimmed mean of M values) method [42] of the edgeR 3.26.6 package [43] in R 3.6.0 (www.r-project.org). Genes with less than one count per million (CPM) in the four biological replicates were discarded. Normalized CPM were used for: 1) a multidimensional scaling analysis; 2) a transcriptome profile analysis with the K-means algorithm. The optimal number of clusters was assessed with the sum of square error method, which showed that six clusters can optimally predict the different transcription patterns; 3) a differential gene expression (DGE) analysis following the general linear model from edgeR package [43, 44]. The differentially expressed genes were determined using "glmFit" function with a Benjamin-Hochberg FDR correction for false positives; 4) Log10 transformation and heatmap generation with heatmap.2 package (https://CRAN.R-project.org/package=gplots).

Finally, predicted *P. protegens* CHA0 coding genes were assigned to Gene Ontology (GO) terms using InterproScan 5.27-66.0 [45]. A GO database was generated with GO.db 3.8.2 package [46]. All DGE genes and transcription profile main clusters genes were used in GO enrichment with GOstats 1.7.4 package in R [47].

*Ortholog analysis*. Whole protein sequences of 97 *Pseudomonas* species (Supplementary Table S2) were compared using OrthoFinder 2.3.3 [48] in an orthologue protein analysis with the default settings. Results were then filtered for chosen categories of proteins. The tree output was represented using FigTree 1.4.4 (http://tree.bio.ed.ac.uk/).
The detailed RNA-seq Script is placed in Supplementary Material 2.

### 11.4. RT-qPCR

In order to verify the RNA-seq results, the expression of selected genes *pap*, *chiD*, *pltA, tpsA2*, *tpsA4* and the PPRCHA0_1961 IS3 transposase gene in the investigated

environments/hosts were quantified using RT-qPCR as described in Supplementary Methods, and Supplementary Tables S3 and S4.

### 11.5. Role of two-partner secretion (TPS) proteins

Domains of *tpsA1, tpsA2, tpsA3* and *tpsA4* encoded proteins were predicted using the HMMER database (www.hmmer.org). The *tpsA* deletion mutants of CHA0 were constructed as described in Supplementary Methods and Supplementary Table S5 and tested for insecticidal activity in feeding assays of 32 or 64 *P. xylostella* larvae and in injection assays of 18 *G. mellonella* larvae as previously described.

### 11.6. Statistics of insect assays

Survival data in the *P. xylostella* feeding and *G. mellonella* injection assays were evaluated using a Log-Rank test of the Survival package of R 3.6.0 (www.r-project.org) with a p-value<0.05.

## 4. Results and Discussion

We analysed the transcriptomic plasticity enabling *P. protegens* CHA0 to colonize lepidopteran larvae and plant roots. These constitute two very different ecological niches in which CHA0 is known to establish pathogenic and beneficial interactions, respectively. We used the CHA0 strain to inoculate wheat roots, feed *P. xylostella* larvae or inject the hemocoel of *G. mellonella* larvae. We analysed the transcriptome of CHA0 after one week on wheat roots, when bacteria had established population sizes of $10^6$ - $10^7$ CFU/mg dry root (Fig. 1b). *P. xylostella* was selected as model to study the progress of insect gut infection 24 and 36 h after feeding. Previous microscopy studies showed that, 1-2 days after feeding *P. xylostella* with treated pellets, CHA0 could only be found in the microvilli of the gut cells and, 3 days after feeding, the insect hemocoel was already heavily colonized by CHA0 [23]. In our study, at 24 h, the larvae showed no disease symptoms yet and were colonized by $10^4$-$10^5$ CFU/mg larvae (Figs. 1a, 1b). At 36 h bacterial loads were 10 times higher, some treated larvae started to die and the remaining were smaller and darker than non-treated larvae, indicating the start of bacterial transmigration from gut to hemocoel (Figs. 1a, 1b). Final mortality assessed in non-extracted *P. xylostella* was 90.6% to 98.4% (Fig. 1a, Supplementary Figure S1). When CHA0 transmigrates into the hemocoel, it causes a systemic infection and eventually kills the insects. Next, we wanted to understand the transcriptomic response in a pure hemolymph environment excluding transmigration factors. Therefore, we injected CHA0 directly into the hemocoel of the bigger insect model *G. mellonella* and extracted RNA 24 h later when the larvae were still alive, non-melanized and inoculant cell numbers had reached $10^5$ CFU/µl hemolymph (Fig. 1b). CHA0 killed 93.3 to 100% of *G. mellonella* larvae within 48 h (Fig. 1a, Supplementary Figure S1). We used the *G. mellonella* injection model because the *P. xylostella* larvae were too small to inject and collect sufficient hemolymph for our RNA sequencing. Previously, CHA0 was shown to multiply and kill different lepidopteran larvae following a similar pattern

[15, 16, 20, 23, 26, 28]. In addition to CHA0 transcriptome remodelling on insect hosts, we analysed the bacterial response to an insect-simulating culture medium without the influence of host immune responses i.e. Grace's insect medium (GIM). Finally, the transcriptome of CHA0 was analysed when growing in a rich culture medium (Lysogeny broth).

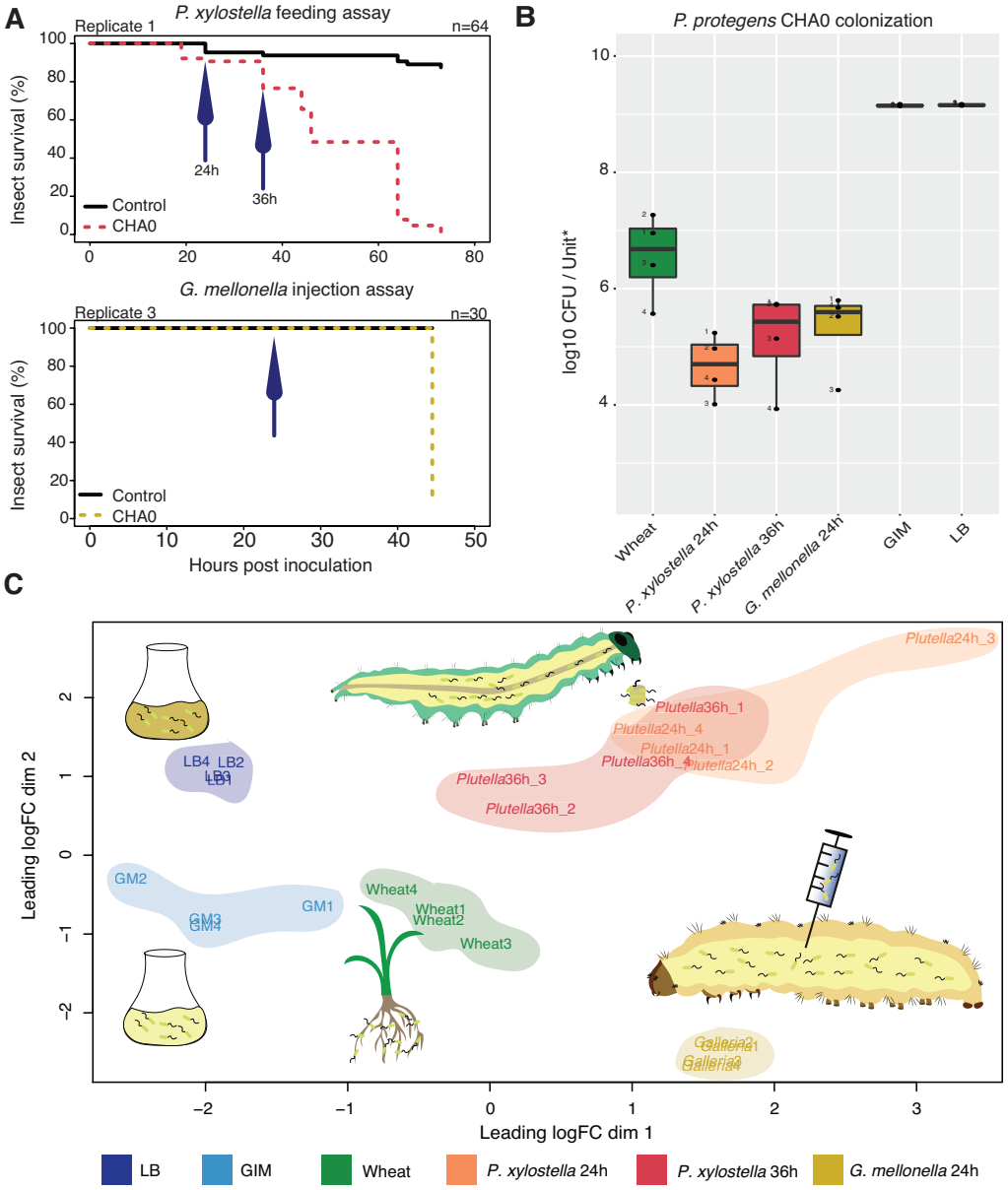


**Figure 1. Toxicity (A), cell density (B) and MDS transcriptome analysis (C) for *Pseudomonas. protegens* CHA0 colonizing different hosts and media.** Wheat = wheat-roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection, LB = lysogeny broth; GIM = Grace's insect medium. A) Survival of *P. xylostella* larvae after exposure to artificial diet pellets spiked with $4 \cdot x\ 10^6$ CHA0 cells (top) and of *G. mellonella* larvae upon injection of $2 \cdot x\ 10^3$ CHA0 cells (bottom). One representative experiment with 64 (*Plutella*) and 30 (*Galleria*) larvae is shown. Time points where insects were sampled for RNA extraction are indicated with arrows. B)

Bacterial densities at collection time points. Boxplots are created from four replicates per environment and show CFU per g of root (wheat), CFU per mg of larvae (24h/36h), CFU per µl hemolymph (*G. mellonella*) and CFU per ml medium (GIM, LB). C): Multidimensional Scaling (MDS) analysis was performed with four replicate CHA0 transcriptomes per environment.

We obtained $10^8$ – $10^9$ reads from RNA Illumina NextSeq sequencing for each sample and quantified expression levels of CHA0 gene models (Supplementary Table S1). We performed a multidimensional scaling analysis to distinguish CHA0 transcriptomes according to the colonized host or culture condition. All transcriptomes were differentially separated except for the *P. xylostella* at 24 h and 36 h due to the differences in infection progression across the biological samples (Fig. 1c).

### 11.1. Pronounced differences between CHA0 transcriptomes during the colonization of wheat roots and insects

The clustering analysis revealed distinct expression profiles for each of the hosts and culture condition (Fig. 2a). This implies that the CHA0 transcriptome changes drastically not only according to the colonized plant or insect, but also according to gut or hemocoel. We found that general metabolic processes and genes related to organic compound biosynthesis were upregulated on wheat roots (Figs. 2b, 3a). Genes related to nucleotide and protein synthesis were downregulated during insect colonization compared to roots (Fig. 3a). This indicates that the bacterium is multiplying and metabolically active on roots using exudates as nutrient sources [49] whereas in the insect gut bacterial proliferation might be restricted. This is in line with the bacterial density determined on roots and in *P. xylostella* samples (Fig. 1b) and previous microscopy studies [23].

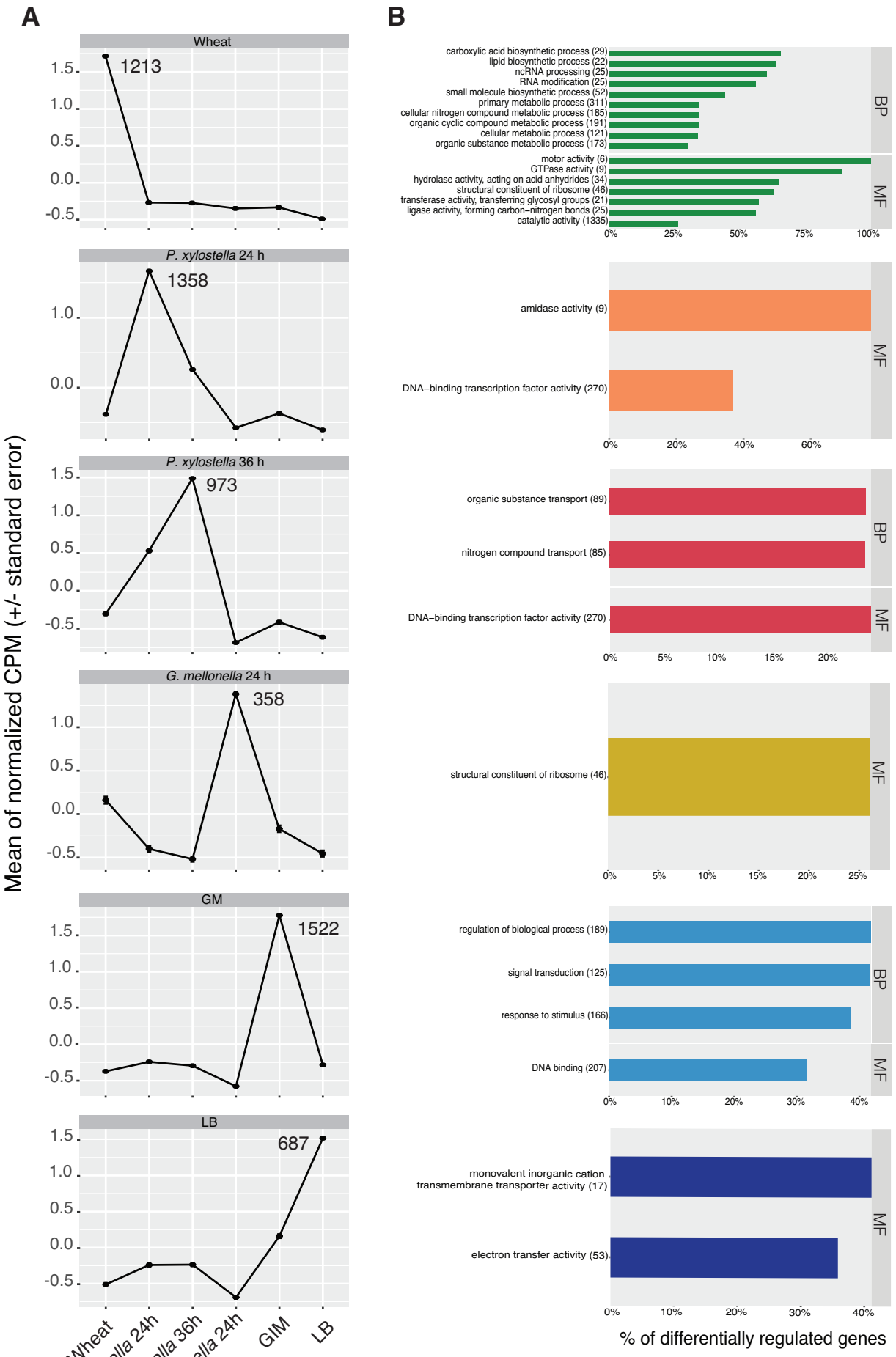
A
B
Mean of normalized CPM (+/- standard error)
Wheat
1213
P. xylostella 24 h
1358
P. xylostella 36 h
973
G. mellonella 24 h
358
GM
1522
LB
687
Wheat
P. xylostella 24h
P. xylostella 36h
G. mellonella 24h
GIM
LB
carboxylic acid biosynthetic process (29)
lipid biosynthetic process (22)
ncRNA processing (25)
RNA modification (25)
small molecule biosynthetic process (52)
primary metabolic process (311)
cellular nitrogen compound metabolic process (185)
organic cyclic compound metabolic process (191)
cellular metabolic process (121)
organic substance metabolic process (173)
motor activity (6)
GTPase activity (9)
hydrolase activity, acting on acid anhydrides (34)
structural constituent of ribosome (46)
transferase activity, transferring glycosyl groups (21)
ligase activity, forming carbon–nitrogen bonds (25)
catalytic activity (1335)
BP
MF
amidase activity (9)
DNA–binding transcription factor activity (270)
organic substance transport (89)
nitrogen compound transport (85)
structural constituent of ribosome (46)
regulation of biological process (189)
signal transduction (125)
response to stimulus (166)
DNA binding (207)
monovalent inorganic cation transmembrane transporter activity (17)
electron transfer activity (53)
% of differentially regulated genes

**Figure 2. Transcription profiles of *Pseudomonas protegens* CHA0 during the colonization of different hosts or environments**. A) Normalized counts per million (CPM) of *P. protegens* CHA0 transcriptomes obtained using the K-means clustering method. Genes clustered in six different groups according to different hosts/media. Standard errors for four biological replicates show small variations among the genes included in each cluster. The number of genes that belong to the main cluster of each transcription profile are indicated. B) Gene ontology (GO) enrichment analysis of the specific genes of each transcription profile main cluster. Significant GO terms for the given set of genes are shown. The number of total genes related to a GO term present in the CHA0 genome is given between brackets and the indicated percentage shows how many of those have higher expression in the specific transcription profile (p-value<0.001). BP: biological process; MF: molecular function. Redundant terms were collapsed. Wheat = wheat roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection, LB = lysogeny broth; GIM = Grace's insect medium.

To successfully reach root zones where exudates are released and to outcompete other organisms, the bacteria need to actively move [49, 50]. Motor activity-related genes were expressed under all conditions but especially upregulated on wheat roots as shown by clustering, differential gene expression (DGE) and heatmap analyses (Figs. 2b, 3a, 3b, Supplementary Figure S2). This confirms the relevance of flagella for wheat root colonization. Interestingly, the *reb* genes required for R-body synthesis were upregulated on wheat roots and repressed in both insect compartments (Fig. 4), which supports the lack of differences in insecticidal activity between Δ*rebB1* mutant and wildtype CHA0 in oral and injection assays [16]. R-bodies disrupt membranes and deliver toxins in several bacterial genera including *Pseudomonas*. The R-bodies are related to *Paramecium* killing and eukaryotic pathogenesis such as the legume *Sebania rostrata* cell-disruption by *Azorhizobium caulinodans* [51–53].

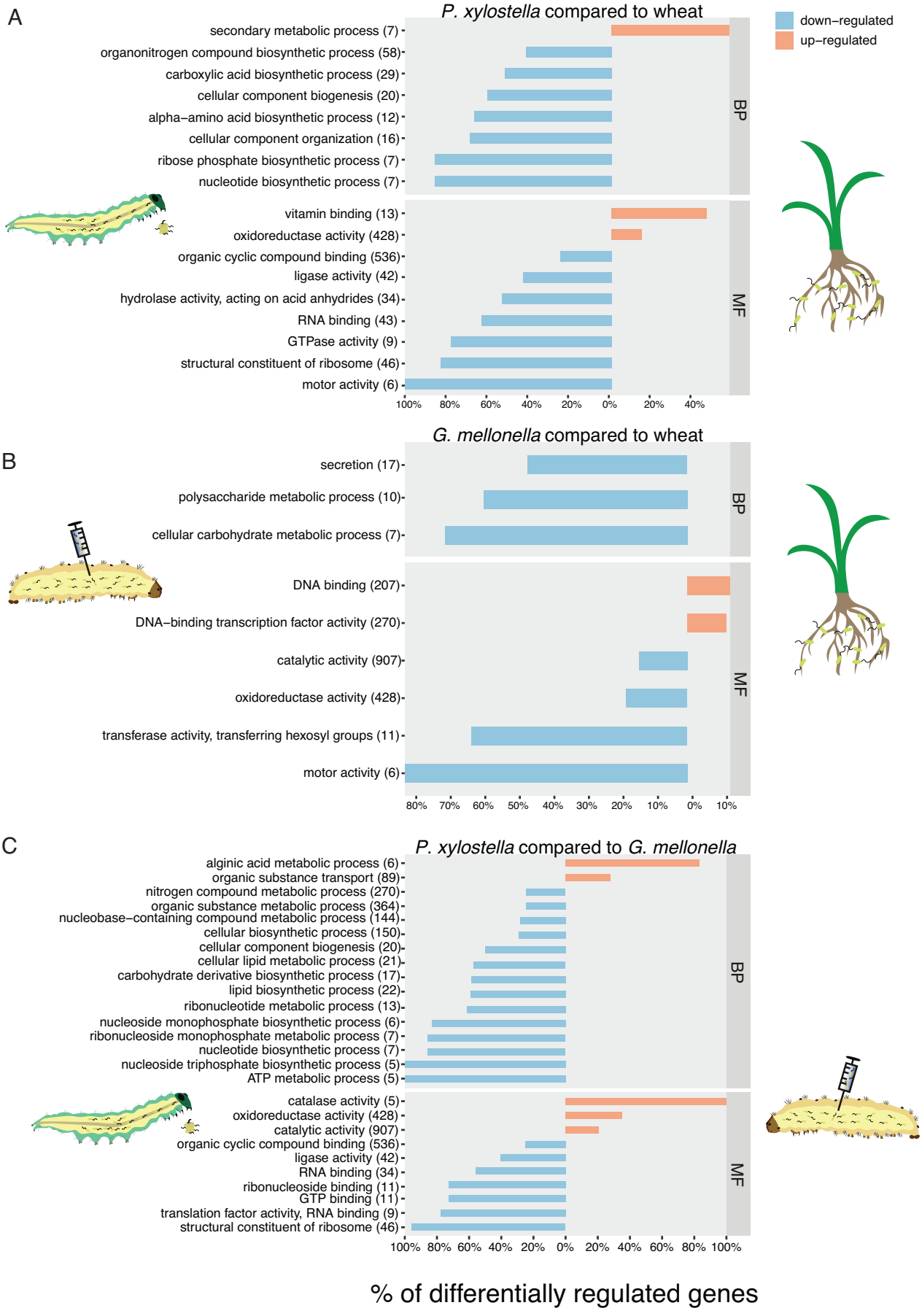
A
P. xylostella compared to wheat
down−regulated
up−regulated
secondary metabolic process (7)
organonitrogen compound biosynthetic process (58)
carboxylic acid biosynthetic process (29)
cellular component biogenesis (20)
alpha−amino acid biosynthetic process (12)
cellular component organization (16)
ribose phosphate biosynthetic process (7)
nucleotide biosynthetic process (7)
vitamin binding (13)
oxidoreductase activity (428)
organic cyclic compound binding (536)
ligase activity (42)
hydrolase activity, acting on acid anhydrides (34)
RNA binding (43)
GTPase activity (9)
structural constituent of ribosome (46)
motor activity (6)
BP
MF
100% 80% 60% 40% 20% 0% 20% 40%
B
G. mellonella compared to wheat
secretion (17)
polysaccharide metabolic process (10)
cellular carbohydrate metabolic process (7)
DNA binding (207)
DNA−binding transcription factor activity (270)
catalytic activity (907)
oxidoreductase activity (428)
transferase activity, transferring hexosyl groups (11)
motor activity (6)
BP
MF
80% 70% 60% 50% 40% 30% 20% 10% 0% 10%
C
P. xylostella compared to G. mellonella
alginic acid metabolic process (6)
organic substance transport (89)
nitrogen compound metabolic process (270)
organic substance metabolic process (364)
nucleobase−containing compound metabolic process (144)
cellular biosynthetic process (150)
cellular component biogenesis (20)
cellular lipid metabolic process (21)
carbohydrate derivative biosynthetic process (17)
lipid biosynthetic process (22)
ribonucleotide metabolic process (13)
nucleoside monophosphate biosynthetic process (6)
ribonucleoside monophosphate metabolic process (7)
nucleotide biosynthetic process (7)
nucleoside triphosphate biosynthetic process (5)
ATP metabolic process (5)
catalase activity (5)
oxidoreductase activity (428)
catalytic activity (907)
organic cyclic compound binding (536)
ligase activity (42)
RNA binding (34)
ribonucleoside binding (11)
GTP binding (11)
translation factor activity, RNA binding (9)
structural constituent of ribosome (46)
BP
MF
100% 80% 60% 40% 20% 0% 20% 40% 60% 80% 100%
% of differentially regulated genes

**Figure 3. Comparisons of transcriptomes of *Pseudomonas protegens* CHA0 between different hosts (A, B) or between different insect compartments (C).** CHA0 transcriptomes colonizing different hosts were analysed by the general linear model pipeline of edgeR package in R. Transcriptomes derived from different hosts were compared pairwise; the reference is always the host shown in the right side of the figure. Total differentially expressed genes for each comparison for each condition were subjected to a GO enrichment analysis. Significant GO terms for the given set of genes are shown. Total genes related to a GO term present in the CHA0 genome are given between brackets and the indicated percentage shows how many of those are differentially expressed in each comparison (p-value<0.001). A) *P. xylostella* vs wheat roots, B) *G. mellonella* hemolymph vs wheat roots, C) *P. xylostella* vs *G. mellonella* hemolymph. BP: biological process; MF: molecular function. Wheat = wheat-roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection.

## 11.2. Specific activities in the insect gut are related to defence against the host immune system and to competition

The gut is a challenging environment for exogenous bacteria as they have to compete with the resident microflora and to overcome the insect immune response e.g. antimicrobial peptides (AMPs) and reactive oxygen and nitrogen species (ROS, RNS) [54]. The *P. xylostella* expression profiles reveal upregulation of several genes, which might help CHA0 to cope with these menaces in the gut. The *P. xylostella* 24 h transcription profile main cluster harbours most of the genes related to amidase activity (Fig. 2). Amidases cleave proteoglycans that trigger host immune response thereby helping bacteria to avoid recognition [54–56].We suggest a similar mechanism in our model. At 36 h after feeding, we found that genes related to the transport of nitrogen and organic substances were specifically upregulated (Fig. 2b, *P. xylostella* 36h). This might be related to the use of nitrogenous compounds emerging from the interaction of ROS and RNS during the insect immune response [57]. The combined evidence from the *P. xylostella* 24/36 h transcriptomic responses compared to wheat root transcriptomes showed upregulation of coding genes of oxidoreductase activity proteins (Fig. 3a). This could be related to the bacterial defence against ROS produced by the insect host. Among the most upregulated genes in the *P. xylostella*-wheat comparison, were *pap* and PPRCHA0_1961 encoding a polyphosphate kinase (PPK) and a predicted “copy-paste” transposase, respectively

(Fig. 4, Supplementary Table 6). PPKs have been related to motility, quorum sensing, biofilm formation and virulence of *P. aeruginosa* and regulation of stress response in *Campylobacter jejuni* [58–62]. We hypothesize that *pap* might play a similar role in CHA0 insect pathogenesis. The PPRCHA0_1961-encoded transposase might perform genomic re-arrangements which are important for bacterial adaptation and pathogenesis as shown for *P. aeruginosa* with another transposable element family [63, 64].

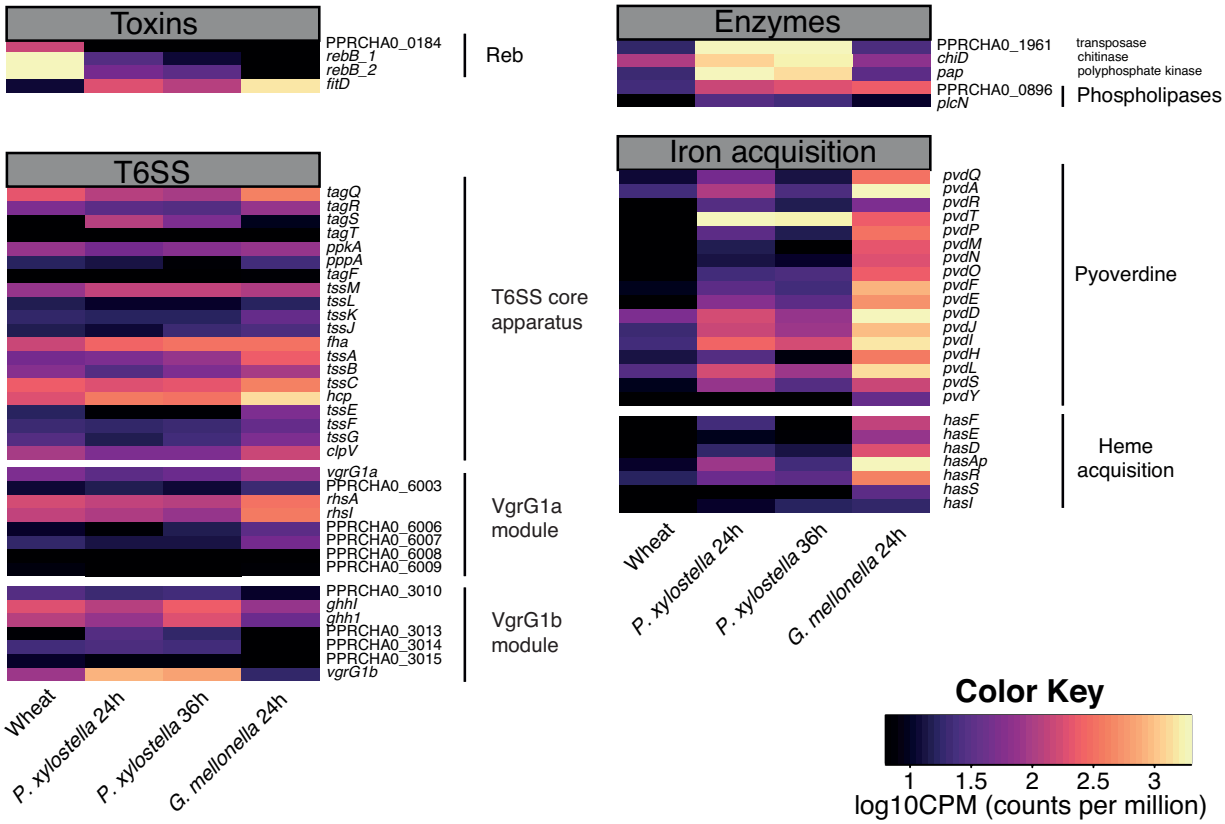


**Figure 4. Heatmap showing the normalized reads (counts per million) for genes related to toxins, type VI secretion system (T6SS), specific enzymes and iron acquisition in *Pseudomonas protegens* CHA0 colonizing different hosts.** Black indicates low expression (less than 10 counts per million reads) and yellow indicates high expression (more than $10^3$ counts per million reads). Wheat = wheat-roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection.

The insect immune response also triggers the production of AMPs that kill invasive bacteria [54]. The dominant short O-antigenic polysaccharide (O-PS) encoded by the OSA cluster confers resistance to insect AMPs in CHA0 [33]. This supports invasion competences

trough oral and systemic insecticidal activity of the bacterium. Of the four CHA0 O-PS clusters [33], OSA and OBC3 (which encodes the major long O-antigen of CHA0) were expressed in all our backgrounds. Interestingly, OBC1 and OBC2 were only expressed in the *P. xylostella* gut (Supplementary Figure S2). It remains unknown whether OBC1 and OBC2 might also play a role in avoiding resistance to or recognition by the insect immune system.

In order to persist, attach and breach the gut epithelium, CHA0 must successfully compete against the resident gut bacteria. CHA0 competes by producing a variety of antimicrobial compounds, which contribute to rhizosphere competence and the suppression of soilborne pathogens [5]. Among the tested hosts, all of the biosynthetic genes involved in the production of the broad-spectrum antimicrobials hydrogen cyanide, 2,4-diacetylphloroglucinol, and pyrrolnitrine were expressed in *P. xylostella* and expressed at low levels on wheat roots (with the exception of 2,4-diactetylphloroglucinol which was highest expressed on roots), but were not expressed in the *G. mellonella* hemolymph. Although the expression of hydrogen cyanide is low in our study, it has been previously shown to influence insect survival when CHA0 is injected in *G. mellonella* hemocoel [29]. Although the expression of pyoluteorin biosynthetic genes was not detected on the roots of any tested plants [37], they were expressed in *P. xylostella* (Supplementary Figure S2). Hence, pyoluteorin might play a more important role in persistence in insect hosts than on plant roots. Combined, our results support that antimicrobials are not only used for competition against microorganisms in the rhizosphere but also during colonization of an insect.

The type VI secretion system (T6SS) has been related to pathogenicity and bacterial competition in the insect gut. In CHA0, the *vgrG1a* and *vgrG1b* genes encoding distinct T6SS spikes and *rhsA* and *ggh1* encoding respective associated effectors with predicted nuclease activity were demonstrated to contribute to invading *Pieris brassicae*. These genes play a role in the ability of CHA0 to compete with the gut microflora and impact on its composition [28].

In our study, these genes were expressed in both insect models (Fig. 4). This underlies the importance of these T6SS components during competitive host invasion [65–67]. Interestingly, the expression of *vgrG1b* and its associated effector gene *ggh1* was higher in the *P. xylostella* gut than in the *G. mellonella* hemolymph. But, we found the opposite for *vgrG1a* and *rhsA* (Fig. 4) indicating that T6SS-mediated competition is important not only in the gut but also at a later infection stage in the hemolymph.

Expression patterns of *pap*, *chiD*, *pltA* and the PPRCHA0_1961 IS3 transposase analysed by qPCR showed the same tendencies as in the RNA-seq with significant differences between environments ($p<0.05$) (Supplementary Figure S3).

### 11.3. Functions underlying transmigration from the gut lumen into the hemocoel

In order to transmigrate from the gut to the hemolymph CHA0 needs to overcome several barriers such as the peritrophic matrix and the gut epithelium. Based on the established expression profiles, we propose the following model of the transmigration process.

Orfamide A, the chitinase and the phospholipase C encoded by *ofaABC*, *chiD* and *plcN* respectively, showed the highest expression in *P. xylostella* when compared to the other environments (Fig. 4, Supplementary Figure S2). In previous studies, CHA0 mutants lacking any of these genes had reduced activity in oral *P. xylostella* feeding assays, but not upon injection into *G. mellonella* hemolymph [16, 29]. This suggests that *ofaABC*, *chiD* and *plcN* are important in the gut infection/transmigration phase. Orfamide A and chitinases were shown to be important in insect pathogenesis even though the exact mechanism remains to be elucidated [29–31]. We hypothesize that orfamide A might be important for adsorption to the peritrophic matrix that lines the gut epithelium and the chitinase might create a passage through this chitinous membrane into the mucus layer. The mammal lung and gut mucus layers are rich in phosphatidylcholine and phosphatidylserine, two major components of eukaryotic

membranes [68]. Phosphatidylserine and phosphatidylcholine are cleaved by PlcN [69] and used as a nutrient source in *P. aeruginosa* [70, 71]. Therefore, we propose that the non-hemolytic PlcN phospholipase might release phospholipids from the gut mucus and weaken the enterocyte membranes in insects. At a later stage of infection, *Pseudomonas* probably use exopolysaccharides to attach to the epithelium as described in chronic lung infections by *P. aeruginosa* [72–75] before they transmigrate to the hemocoel. We identified the protein functions associated to alginic acid metabolism and the transport of organic compounds being differentially regulated when comparing *P. xylostella* to *G. mellonella* (Fig. 3c) supporting our model. The CHA0 exopolysaccharide gene clusters *alg*, *psl*, *pga* and *pel* were barely expressed in the *G. mellonella* hemolymph but were upregulated on roots and in *P. xylostella.* This upregulation could play a role in attachment to the insect gut surface (Supplementary Figure S2).

Once the bacteria have passed through the peritrophic matrix and are attached to the gut, they need to disrupt the epithelial cells to create a passage to the hemocoel. Based on our transcriptomic evidence, we found that two-partner secretion systems (TPS) may be involved in this process. TPS of Gram-negative bacteria consist of a B transporter and an A effector protein, e.g. an adhesin, hemolysin, or exolysin [76]. TPS secreted toxins were identified in different Gram-negative bacteria e.g. in *P. aeruginosa*, *Serratia marcescens*, *Proteus mirabilis*, *Haemophilus influenza*, and entomopathogenic bacteria such as *Photorhabdus luminescens* and *P. entomophila* [76–81]. TPS are related to cellular adhesion, pore formation and competition across bacterial species [76, 82], host tissue damage [83], cell-junctions cleavage in *P. aeruginosa* PA7 [76, 81, 84], and macrophage pyroptosis in *P. aeruginosa* PA7, *P. entomophila* L48, *P. putida* KT2440 and *P. protegens* CHA0 by ExlA-like proteins [80].

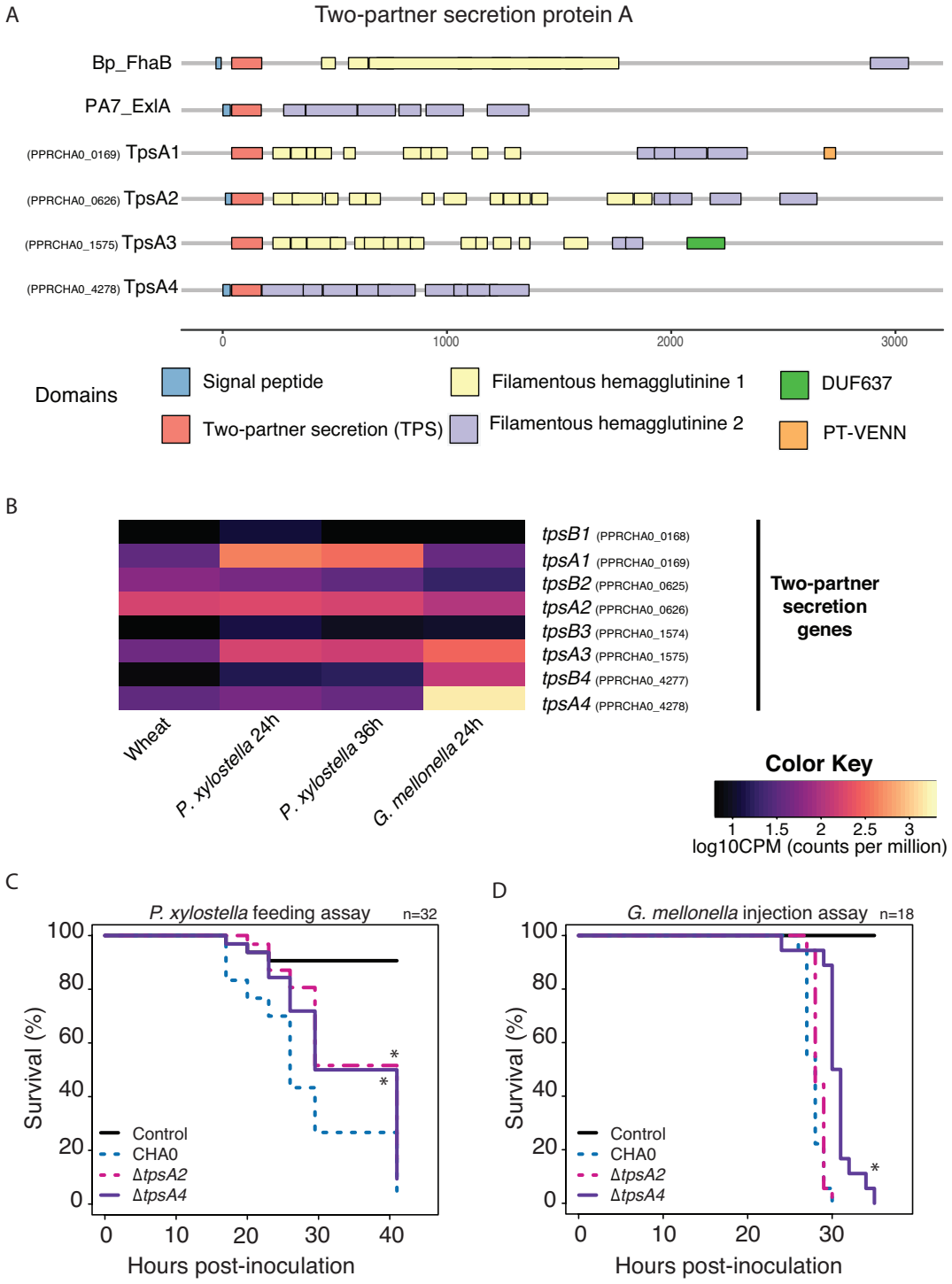


**Figure 5. Two-partner secretion (TPS) systems in *Pseudomonas protegens* CHA0: domain analysis (A), expression profiles in relation to different hosts (B) and contribution to insecticidal activity (C).** A) Domain analysis of the secreted protein with HMMER database comparing *Bordetella pertussis* protein FhaB, *Pseudomonas aeruginosa* PA7 protein ExlA and *P. protegens* CHA0 proteins TpsA1, TpsA2, TpsA3 and TpsA4 (PPRCHA0_0168-169, PPRCHA0_0625-0626, PPRCHA0_1574-1575, and PPRCHA0_4277-4278, respectively). The signal peptide and TPS domains are used to interact with the transporter protein for membrane translocation; the filamentous hemagglutinin 1 attaches to the host-cell and the filamentous hemagglutinin 2 translocates the PT-VENN domain into the host; DUF637 is common to hemagglutinins but its function is still

unclear [82]. B) Heatmap showing the normalized expression values for genes related to the four complete two-partner secretion systems in *P. protegens* CHA0 colonizing different hosts. Wheat = wheat-roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection. C) Survival of *P. xylostella* larvae after exposure to artificial diet pellets spiked with 4 x $10^6$ cells of CHA0 wild type, or its *tpsA2* or *tpsA4* deletion mutants. Thirty-two 2$^{nd}$ instar larvae were used per bacterial strain. D) Survival of *G. mellonella* larvae after injection of 2 x $10^3$ cells into the hemocoel. Eighteen 7$^{th}$ instar larvae were used per bacterial strain. One experiment of each is shown and two more P. *xylostella* feeding and one *G. mellonella* injection are shown in Supplementary Figure S4. Asterisks indicate significant differences of mutants to the wildtype (log-rank test, $P$<0.05).

The CHA0 genome harbours four predicted complete TPS named *tpsBA1-4* encoding TpsBA1-4 (Fig. 5; PPRCHA0_0168-169, PPRCHA0_0625-0626, PPRCHA0_1574-1575, and PPRCHA0_4277-4278). TPSA1-4 effector proteins share between 39.3% and 59.5% amino acid identity with the ExlA protein of *P. aeruginosa* PA7 (Supplementary Table S7). Similarly to PA7, ExlA and FhaB of *Bordetella pertussis*, CHA0 TpsA1-4 have a N-terminal TPS domain followed by several copies of filamentous-haemagglutinine domains (FLH1 and 2 domains, Fig. 5) [81, 82]. In addition, TpsA1 also has a pre-toxin VENN domain (Fig. 5a) commonly involved in cell-cell interactions [82, 85]. While the domain structure of TpsA4 is highly similar to PA7 ExlA, TpsA1-3 contain several predicted FLH1 repeats in addition to the FLH2 domains (Fig. 5).

The four *tpsA* genes showed very different expression profiles across the examined environments (Fig. 5b). While *tpsA2* was equally expressed in all examined hosts, the other three variants were upregulated in insects when compared to wheat roots. *tpsA3* was similarly expressed in both insect models, in comparison to *tpsA1* which was upregulated during *P. xylostella* infection and *tpsA4* which was highly upregulated in *G. mellonella* hemolymph. Interestingly, *tpsA4* was among the 20 most upregulated genes in *G. mellonella* when compared to *P. xylostella* (Supplementary Table S6).

In order to assess the importance of TpsA toxins for insect infection, we tested *tpsA* knockout mutants in feeding assays with *P. xylostella* and in injection assays with *G.*

*mellonella*. The deletion of *tpsA4,* which was highly expressed in an insect background but not on roots, resulted in significantly reduced insecticidal activity in two out of three feeding experiments. A deletion mutant of *tpsA2,* which was expressed in all tested hosts, led to significantly reduced insecticidal activity in one out of three feeding experiments ($p<0.05$) (Figs. 5c, Supplementary Figure S4a, b). Additionally, a *tpsA4* deletion mutant showed significantly slower mortality when injected into the *G. mellonella* hemocoel in two experiments ($p<0.05$) (Figs. 5d, Supplementary Figure S4c). Furthermore, we confirmed the expression patterns of *tpsA2* and *tpsA4* by qPCR showing the same tendencies as found in the transcriptomic analyses with significant differences between environments ($p<0.05$) (Supplementary Figure S3).

The functions of the CHA0 TPS-secreted toxins are still unknown, but these toxins may play similar roles as the *P. aeruginosa* toxin ExlA. TpsBA1-3 may be involved in gut colonization, adhesion to tissues, bacterial competition and the disruption of the gut epithelium. The high expression of TpsBA4 in the hemolymph suggests that the protein could be involved in the defence against insect immune reactions e.g. by triggering hemocyte cell death. Our model of TPS interactions are supported by the findings that CHA0 mutants lacking t*psA4* are largely impaired in macrophage killing [80].

### 11.4. The insect hemocoel is more permissive for rapid proliferation than the gut

During the first phase of gut infection, CHA0 shows limited growth and metabolism. In contrast, we found an upregulation of structural ribosome constituents and nucleic acid synthesis in the *G. mellonella* hemolymph (Fig. 2b, 3c). The pairwise comparison revealed a general upregulation of genes involved in proliferation and metabolic activities in the *G. mellonella* hemolymph compared to the *P. xylostella* gut (Fig. 3c). In contrast, oxidoreductase and catalase activity were downregulated compared to *P. xylostella* and wheat (Figs. 3c, 3b).

These differences indicate that the hemolymph is a less stressful environment and that CHA0 has enough nutrients allowing rapid proliferation leading to the systemic infection and ultimately death of the insect. However, oxidoreductase functions were upregulated in the *G. mellonella* hemolymph when compared to the hemolymph mimicking Grace's Medium (Supplementary Figure S5, Table S6). This may be related to defences against insect immune responses. Furthermore, the bacteria are challenged by iron deprivation forcing a strong upregulation of pyoverdine synthesis and heme-acquisition related genes (Fig. 4, Supplementary Table S6). Pathogenic bacteria need siderophores such as pyoverdine and heme-acquistion systems to acquire the essential iron from iron-binding proteins in the gut lumen, the hemolymph and the fat body [86–88].

The Fit insect toxin substantially contributes to insect killing in systemic infections by CHA0 and other *P. protegens*/*P. chlororaphis* [15, 17, 20]. It possibly interferes with the activity of hemocytes as was shown for the related apoptotic toxin Mcf of *P. luminescens,* ([89, 90]. Previously, Fit was shown to be only produced in the insect hemolymph but not on plant roots using a mCherry-labelled FitD [20]. We can show now that *fitD* was among the 20 most upregulated genes in the *G. mellonella*-wheat comparison (Fig. 4; Supplementary Table S6). Our study further shows that the maximal expression of *fitD* occurs in the hemolymph but that the upregulation is initiated when the bacteria colonize the insect gut (Fig. 4).

### 11.5. Genus-wide comparative genomics to retrace the evolutionary origins of *Pseudomonas protegens* CHA0 pathogenicity factors

We compared the full protein sequence of CHA0 and 96 pseudomonads using ortholog analyses (Supplementary Figure S6). We analysed phylogenetic groups harbouring strains showing plant beneficial interactions as well as and plant-, insect- and human pathogenic abilities (Supplementay Table S1). *P. protegens*/*P. chlororaphis* subgroups possess a set of

specific traits absent in other *Pseudomonas* groups (Figure 6, Supplementary Figure S6). We investigated whether CHA0 genes responding to lifestyle changes were common to other pathogenic and beneficial *Pseudomonas*. We focussed on genes with host or insect compartment specific expression that have emerged from this study and/or have been shown to contribute to insecticidal activity in earlier studies [16, 28] i.e. specific exoenzymes, exopolysaccharides, T6SS modules and toxins.

Some genes with lifestyle specific expression patterns are distinct of the *P. protegens* subgroup including the transposase PPRCHA0_1961, Vgr1a elements *e.g.* the Rhsl effector [28] and TpsB1 (Fig. 6). This suggests that these genes were recently acquired by *P. protegens* or were lost in all other groups. We propose that some of these genes are specific for insect interactions. However, most functions in insecticidal activity are shared by the *P. protegens* and *P. chlororaphis* subgroups. Intriguingly, some of these traits are also present in the phylogenetically distant *P. aeruginosa* group [1, 2] harbouring animal and human pathogens. Among these functions, we found a chitinase, the phospholipase PlcN, proteins related to the production of the exopolysaccharide Pel, T6SS components of the Vgr1A and Vgr1b modules, the Reb toxins or some proteins related to stress-response. The presence of these insect interaction-related CHA0 traits in very distant species such as *P. aeruginosa* (Fig. 6) suggests that either these are ancestral functions and were lost repeatedly during the evolution of *Pseudomonas* or that they have been independently acquired. Interestingly, the entomopathogen *P. entomophila* [24] lacks several of the CHA0 functions shared with the *aeruginosa* group including Pel, Psl, the Vgr1a and b elements [28], and some stress-related proteins (Fig. 6). *P. chlororaphis* and *P. protegens* with the ability to colonize plant and insect environments seem to have a very distinct toolbox when compared to the rest of the analysed species (Fig. 6, Supplementary Figure S6).

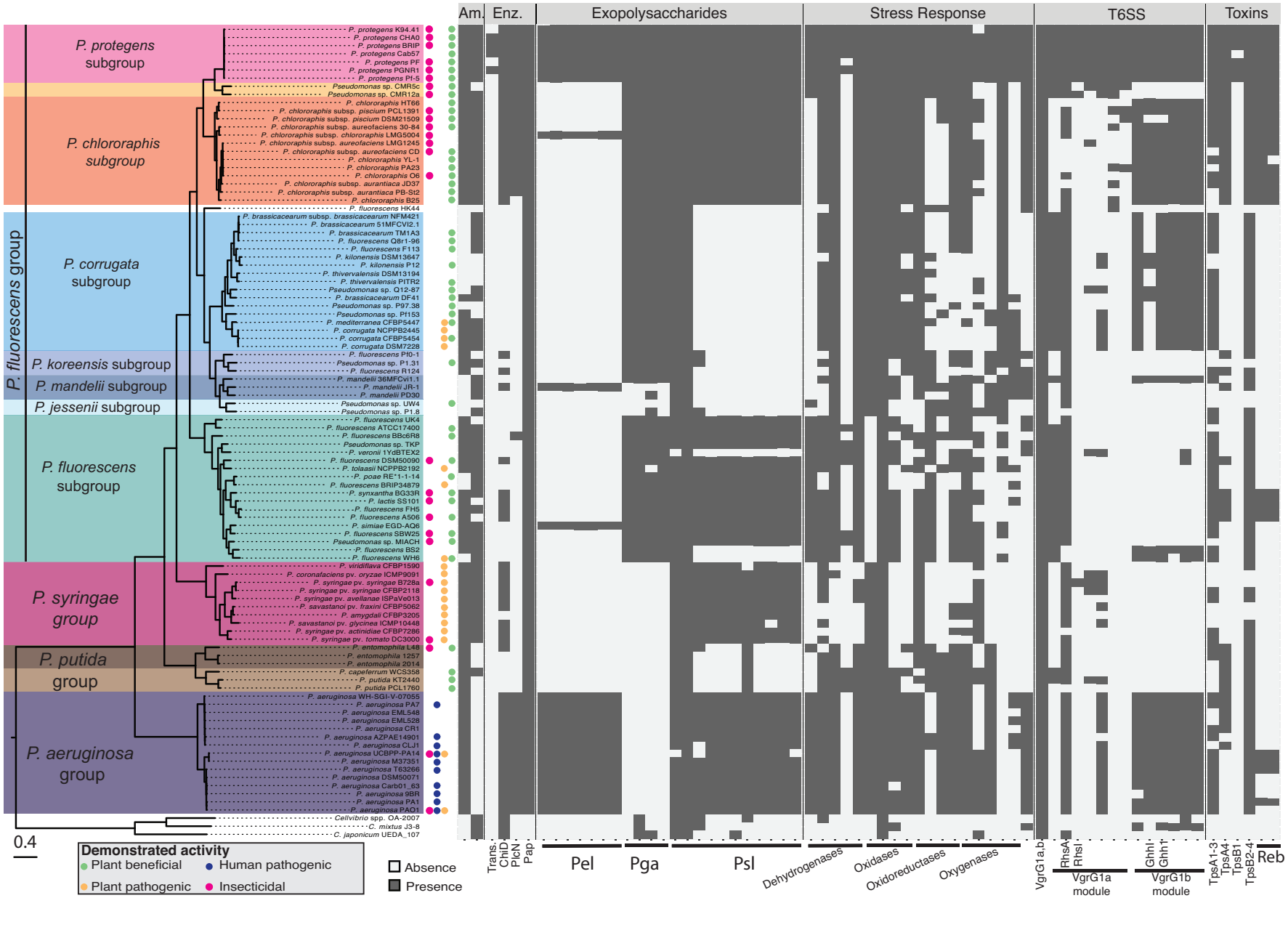
P. fluorescens group
P. protegens subgroup
P. chlororaphis subgroup
P. corrugata subgroup
P. koreensis subgroup
P. mandelii subgroup
P. jessenii subgroup
P. fluorescens subgroup
P. syringae group
P. putida group
P. aeruginosa group
P. protegens K94.41
P. protegens CHA0
P. protegens BRIP
P. protegens Cab57
P. protegens PF
P. protegens PGNR1
P. protegens Pf-5
Pseudomonas sp. CMR5c
Pseudomonas sp. CMR12a
P. chlororaphis HT66
P. chlororaphis subsp. piscium PCL1391
P. chlororaphis subsp. piscium DSM21509
P. chlororaphis subsp. aureofaciens 30-84
P. chlororaphis subsp. chlororaphis LMG5004
P. chlororaphis subsp. aureofaciens LMG1245
P. chlororaphis subsp. aureofaciens CD
P. chlororaphis YL-1
P. chlororaphis PA23
P. chlororaphis O6
P. chlororaphis subsp. aurantiaca JD37
P. chlororaphis subsp. aurantiaca PB-St2
P. chlororaphis B25
P. fluorescens HK44
P. brassicacearum subsp. brassicacearum NFM421
P. brassicacearum 51MFCVI2.1
P. brassicacearum TM1A3
P. fluorescens Q8r1-96
P. fluorescens F113
P. kilonensis DSM13647
P. kilonensis P12
P. thivervalensis DSM13194
P. thivervalensis PITR2
Pseudomonas sp. Q12-87
P. brassicacearum DF41
Pseudomonas sp. P97.38
Pseudomonas sp. Pf153
P. mediterranea CFBP5447
P. corrugata NCPPB2445
P. corrugata CFBP5454
P. corrugata DSM7228
P. fluorescens Pf0-1
Pseudomonas sp. P1.31
P. fluorescens R124
P. mandelii 36MFCvi1.1
P. mandelii JR-1
P. mandelii PD30
Pseudomonas sp. UW4
Pseudomonas sp. P1.8
P. fluorescens UK4
P. fluorescens ATCC17400
P. fluorescens BBc6R8
Pseudomonas sp. TKP
P. veronii 1YdBTEX2
P. fluorescens DSM50090
P. tolaasii NCPPB2192
P. poae RE*1-1-14
P. fluorescens BRIP34879
P. synxantha BG33R
P. lactis SS101
P. fluorescens FH5
P. fluorescens A506
P. simiae EGD-AQ6
P. fluorescens SBW25
Pseudomonas sp. MIACH
P. fluorescens BS2
P. fluorescens WH6
P. viridiflava CFBP1590
P. coronafaciens pv. oryzae ICMP9091
P. syringae pv. syringae B728a
P. syringae pv. syringae CFBP2118
P. syringae pv. avellanae ISPaVe013
P. savastanoi pv. fraxini CFBP5062
P. amygdali CFBP3205
P. savastanoi pv. glycinea ICMP10448
P. syringae pv. actinidiae CFBP7286
P. syringae pv. tomato DC3000
P. entomophila L48
P. entomophila 1257
P. entomophila 2014
P. capeferrum WCS358
P. putida KT2440
P. putida PCL1760
P. aeruginosa WH-SGI-V-07055
P. aeruginosa PA7
P. aeruginosa EML548
P. aeruginosa EML528
P. aeruginosa CR1
P. aeruginosa AZPAE14901
P. aeruginosa CLJ1
P. aeruginosa UCBPP-PA14
P. aeruginosa M37351
P. aeruginosa T63266
P. aeruginosa DSM50071
P. aeruginosa Carb01_63
P. aeruginosa 9BR
P. aeruginosa PA1
P. aeruginosa PAO1
Cellvibrio spp. OA-2007
C. mixtus J3-8
C. japonicum UEDA_107
0.4
Demonstrated activity
Plant beneficial
Human pathogenic
Plant pathogenic
Insecticidal
Absence
Presence
Am.
Enz.
Trans.
ChiD
PlcN
Pap
Exopolysaccharides
Pel
Pga
Psl
Stress Response
Dehydrogenases
Oxidases
Oxidoreductases
Oxygenases
T6SS
VgrG1a,b
RhsA
RhsI
VgrG1a module
GhhI
GhhT
VgrG1b module
Toxins
TpsA1-3
TpsA4
TpsB1
TpsB2-4
Reb

**Figure 6. Orthologue protein analysis examining the presence of *Pseudomonas protegens* CHA0 factors associated with insect pathogenicity across different phylogenetic *Pseudomonas* groups.** A comparison of the full *in silico* proteomes of 97 pseudomonads belonging to phylogenetic groups harboring insect pathogenic, human pathogenic, plant pathogenic and plant beneficial strains (groups and subgroups as defined by Hesse et al.,[2]) was performed and is shown in Supplementary Figure S6. Here, we show the distribution of selected CHA0 traits investigated in this study in ecologically different *Pseudomonas* strains. Strains with described activity are marked in: pink for insecticidal activity (oral or injectable); dark-blue for human pathogenic activity; orange for plant pathogenic activity; green for plant-beneficial activity. Abbreviations: Am.: amidases; Enz.: enzymes; Trans.: transposase

# 5. Conclusions

*P. protegens* and *P. chlororaphis* are bacterial species with multifaceted lifestyles as they can easily switch between plant and insect hosts. Our analyses of the *P. protegens* CHA0 transcriptomes across plant, insect and specific culture medium conditions significantly enhance our understanding of the shared and specific functions deployed across host-associated lifestyles. We have also shown how different functions are modulated over the course of an insect infection. Our results show that CHA0 deploys distinct toolsets to colonize plant-roots, the insect gut and the insect hemocoel with specific expression in some environments (e.g. flagella on roots or the toxins TpsA4 and Fit in the insect hemocoel). In contrast, we also discovered that antimicrobial metabolites, the T6SS and exopolysaccharides serve as weapons or colonization factors across multiple environments. Based on the results presented here and our previous studies on insecticidal traits, we propose a comprehensive insect colonization and pathogenesis model for *P. protegens* CHA0 as summarized in Figure 7. We finally show that some key insect pathogenicity factors are conserved across *Pseudomonas* groups, while other factors are patchily distributed in *P. protegens* or *P. protegens*/*chlororaphi*s suggesting distinct evolutionary origins.

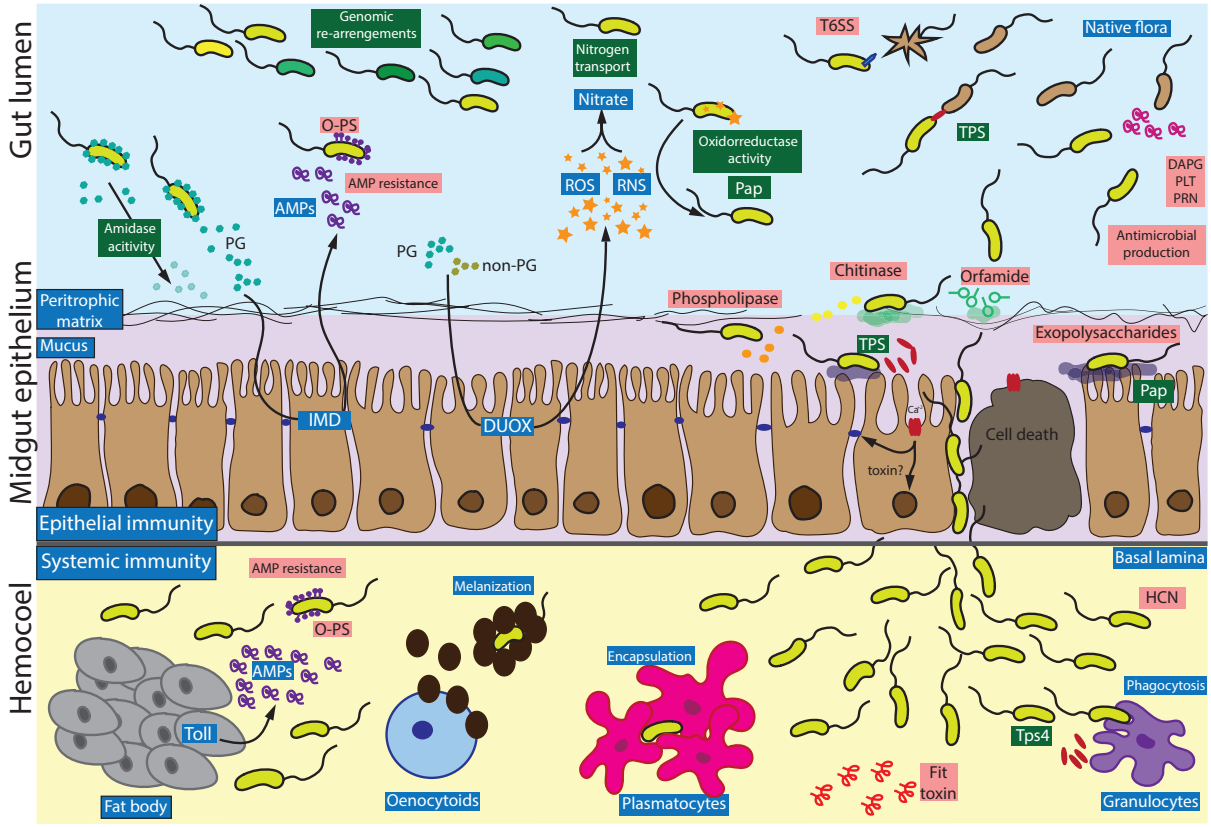


**Figure 7. Proposed pathogenesis model of *P. protegens* CHA0 colonizing Lepidoptera insect pests after oral infection**. In the proposed pathogenesis model, the insect immune response is marked in blue, CHA0 factors emerging from this study in dark green and factors shown to be in involved in *P. protegens* CHA0 insecticidal activity in previous studies in pink. As described previously by Engel and Moran [54], in the event of a pathogen invasion, the insect will detect the presence of proteoglycans or other bacterial components that will trigger the immune response. The gut epithelium activates the production of reactive oxygen and nitrogen species (ROS and RNS) through the DUOX membrane oxidases and antimicrobial peptides (AMPs) through the IMD pathway. If the signal reaches the insect fat body, the Toll pathway will activate the production of AMPs as well [54, 91]. We hypothesise the following infection process: CHA0 is taken up by an insect feeding on plant colonized by the bacterium. In the gut, the bacterium faces the first line of the insect defence and has to compete with the resident microflora. Amidase activity degrading proteoglycan residues from the cell wall helps *P. protegens* CHA0 to avoid recognition by the immune system. The bacterium further uses oxidoreductases and the Pap protein to protect itself against reactive oxygen and nitrogen species. Nitrogen transporters might capture nitrogenous compounds resulting from the interaction of RNS and ROS. In order to better survive this adverse and stressful environment, it is possible that *P. protegens* CHA0 activates transposases for genomic re-arrangements in order to increase its genomic variability. The bacterial cells can resist AMPs thanks to the O-polysaccharide conformation of its surface [33]. CHA0 also produces antimicrobial compounds as shown here and in Flury et al., [29] and it uses the type VI secretion system (T6SS) to fight the microflora of the insect or other ingested bacteria. For breaching the gut epithelium, we propose the following scenario: To adhere to the surface of the peritrophic matrix, CHA0 uses the cyclic lipopeptide surfactant orfamide A [29]. Then, the chitinase disrupts the chitinous peritrophic matrix [16]. *P. protegens* CHA0 may use the phospholipase PlcN to release nutrients from the mucus layer or to damage the epithelial cells [16, 92] and exopolysaccharides to establish in the epithelium [75].

Subsequently, the production of different two-partner secretion proteins (TPS) triggers host cell death and disrupts the cadherine junctions between epithelial cells. This will allow the bacteria to transmigrate into the hemocoel. Here CHA0 has to resist phagocytosis by granulocytes, encapsulation by plasmatocytes, melanine coating by oenocytoids and AMPs produced by the fat body [21, 93]. To fight the immune cells, CHA0 might use the FitD toxin [33], hydrogen cyanide (HCN) [29] and the TpsA proteins which, in combination with the bacterial multiplication, will finally lead to the death of the insect.

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## 7. Conflict of Interest

The authors declare no conflict of interest.

## 8. Sources of support

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## 9. Data availability

The generated RNAseq datasets were deposited on the NCBI Short Read Archive under the BioProject ID PRJNA595077.

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# 11. Supplementary information

## 11.1. Supplementary Methods

### Wheat-root colonization assay

The assay described in de Werra et al., 2008 was used with some modifications. Spring wheat seeds of the variety Rubli (Delley Samen und Pflanzen AG, Delley, Switzerland) were surface disinfected for 30 min with 1.4% NaClO (vol/vol) and subsequently rinsed with autoclaved distilled $H_2O$. Clean seeds were pre-germinated on 1.5% agar plates for two days and then transferred to CYG seed germination pouches (18 cm high by 16.5 cm wide, Mega International, West St. Paul, Mn, U.S.A.), three seeds per pouch. 1 ml of a suspension containing *P. protegens* CHA0 cells ($OD_{600} \approx 0.125 \approx 10^8$ cell/ml) was inoculated onto the seeds. For each replicate 33 pouches with 3 seeds each were prepared. Plants were grown at 22 °C and 70% relative humidity with a 16/8 h day (270 µmol $m^{-2}s^{-1}$)/ night cycle. After one week, roots of 99 plants were harvested in batches of 9 plants, placed into 50 ml 0.9% NaCl and shaken at 400 rpm for 20 min. Pellets were pooled in 50 ml 0.9% NaCl. Two-hundred-fifty µl of the resulting suspension were used to assess bacterial colonization by plating dilution series onto King' B [1] plates supplemented with three antibiotics (= KB+++): ampicillin, 40 µl $ml^{-1}$; chloramphenicol, 13 µl $ml^{-1}$; and cycloheximide, 100 µl $ml^{-1}$ [2] The remaining suspension was centrifuged and the pellet containing bacteria frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Four independent replicates were prepared.

### *P. xylostella* feeding assay

*P. xylostella* eggs were obtained from Syngenta Crop Protection AG (Stein, Switzerland). Insects were kept before and during the experiment at 25 °C, 60% humidity and 16h - 8h day (162 µmol $m^{-2}s^{-1}$)/night cycle. For the feeding assays, 185 second instar larvae were placed individually into wells of 128-well bioassay trays (Frontier Agricultural Sciences, Delaware, USA) to avoid cannibalism. Each well contained a wetted filter paper and a piece of diet pellet spiked with 10 µl NaCl 0.9% (control) or 10 µl of a suspension of *P. protegens* CHA0 cells ($OD \approx 0.5 \approx 4 \times 10^8$ cfu/pellet) resulting in $4 \times 10^6$ cfu per pellet. The artificial diet used for the experiments was prepared by boiling 7.5 g of agar in 500 ml of distilled $H_2O$ for 1 min. Then 50 g of Adapta Bio-Dinkel cereal (Hero Baby, Switzerland), 1 effervescent vitamin pill

(Santogen Gold, Switzerland), 15.5 g yeast extract (Difco, MI, USA), 7.5 g casaminoacids (Difco, MI, USA), 0.25 g cholesterol (Sigma Aldrich, MO, USA), 0.5 ml corn oil (Coop, Switzerland) were added and homogenized with a blender. The homogenate was poured in Petri dishes up to 2 mm of thickness and the pellets were further cut in 4 mm diameter pieces with a cork borer [3]. Sixty-three to sixty-five larvae per treatment were kept for assessing survival by repeated poking and the rest was prepared for RNA extraction as follows.

One-hundred-twenty larvae were collected 24 h and 36 h after feeding and surface disinfected by washing in ethanol 70% (20 s) and rinsing with sterile distilled $H_2O$ and 0.05% SDS (20 s) twice. The effectiveness of this method was evaluated in previous studies [4]. Portions of 30 surface disinfected larvae were homogenized in 1 ml NaCl 0.9% with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). All homogenates were pooled. Two hundred fifty µl of the homogenate were used to assess bacterial colonization as described above. The remaining sample was centrifuged at 7500 rpm for 5 min, frozen in liquid $N_2$ and preserved at -80°C until RNA extraction. Four replicate samples were prepared over time, only from batches (feeding assays) with final mortality higher than 90%.

### *G. mellonella* injection assay

Four replicates of last (seventh) instar *G. mellonella* larvae (Hebeisen Fishing, Zürich, Switzerland) were injected with 10 µl of a suspension containing 2 x $10^5$ *P. protegens* CHA0 cells or 10 µl of sterile 0.9% NaCl solution with a repetitive dispensing Tridak Stepper (Intertronic, Oxfordshire, UK). Groups of 10 larvae were kept together in a Petri dish at 24 °C in the dark until the end of the experiment. One part of the larvae, i.e. 30 in replicates 1 to 3 or 50 in replicate 4, were used to assess survival. Larvae were considered dead when they did not react anymore to repeated poking with a tip. The rest of the larvae (55) in each replicate were used for preparing samples for RNA extraction. After 24 h, these 55 larvae were surface disinfected as described for *P. xylostella* above. One leg was cut and the hemolymph was gently squeezed in an Eppendorf tube. For each larva, 1 µl of the hemolymph was used to assess bacterial colonization as described above. The harvested hemolymph from all 55 larvae was pooled, immediately frozen in liquid $N_2$ and stored at -80 °C until RNA extraction. Four replicates were prepared over time.

**RT-qPCR**

RNA was transformed into cDNA by RevertAid First Strand cDNA Synthesis Kit™ (Thermo Scientific, MA, USA) and used in a quantitative PCR reaction performed with Eva Green™ (Biotium, CA, USA) in a LightCycler480 (Roche, Switzerland). Conditions and primers of the qPCR are reported in Supplementary Table S3 and S4. All four biological replicates were tested in triplicates. The amplification efficiency was further checked by the LinRegPCR software [5] to discard data points with an efficiency under 1.8. Each data point was adjusted with its individual efficiency. The expression of each gene was corrected with the 16S rRNA gene as reference and normalized to the expression of the respective gene on wheat roots according to Pfaffl model [6]. Wheat root was used as the calibrator sample to assess the differences between colonizing insects and roots. Mean expression differences were assessed by a Kruskal Wallis test and a pair-wise comparison (Dunn's posthoc test, $p<0.05$) was performed comparing each background to wheat in R 3.6.0 (www.r-project.org).

**Construction of *tpsA* mutants.**

The *tpsA* deletions mutants of *P. protegens* CHA0 i.e. Δ*tpsA2* (PPRCHA0_0626) and Δ*tpsA4* (PPRCHA0_4278) were generated using the allelic replacement technique with the I-SceI system from the suicide vector pEMG [7] as described in Kupferschmied et al. [8] with the primer pairs listed in Supplementary Table S5.

## 11.2. Supplementary Figures

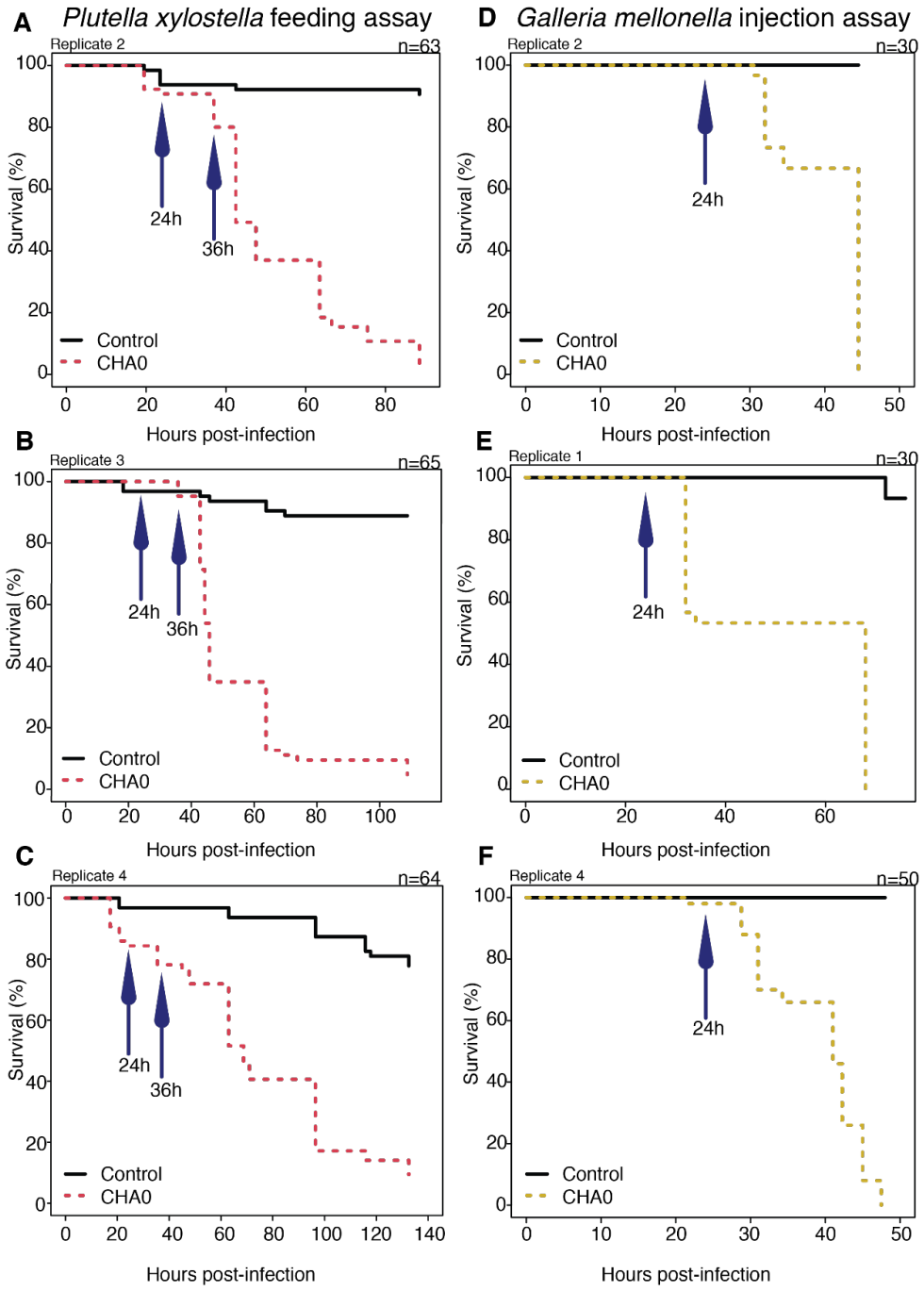


**Figure S1. Survival of *Plutella xylostella* (left panels) and *Galleria mellonella* (right panels) larvae after inoculation with *P. protegens* CHA0.** A-C) *P. xylostella* larvae were exposed to artificial diet pellets spiked with 4·x $10^6$ CHA0 cells. D-F) Seventh instar *G. mellonella* larvae were injected with 2·x $10^3$ CHA0 cells. At the time points indicated by arrows a part of the larvae were used for RNA extraction. The replicate number and the numbers of larvae used for calculation of the survival curves are indicated on top of the panels.

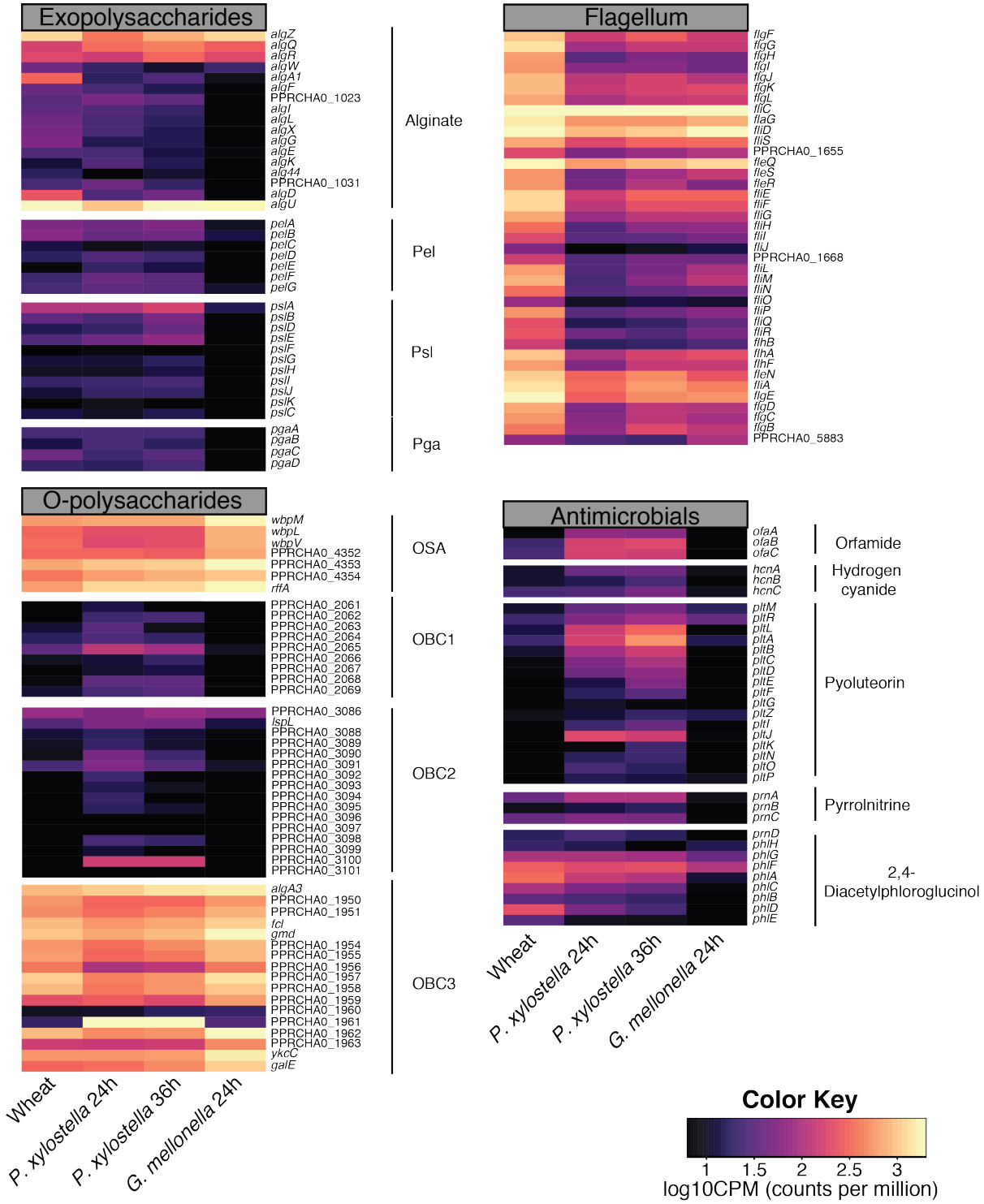


**Figure S2. Heatmap showing the normalized reads (counts per million) for genes related to exopolysaccharides, o-polyaccharides, flagellum and antimicrobials in *P. protegens* CHA0 colonizing different hosts.** Black indicates low expression (less than 10 counts per million reads) and yellow indicates high expression (more than $10^3$ counts per million reads). Wheat = wheat-roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection.

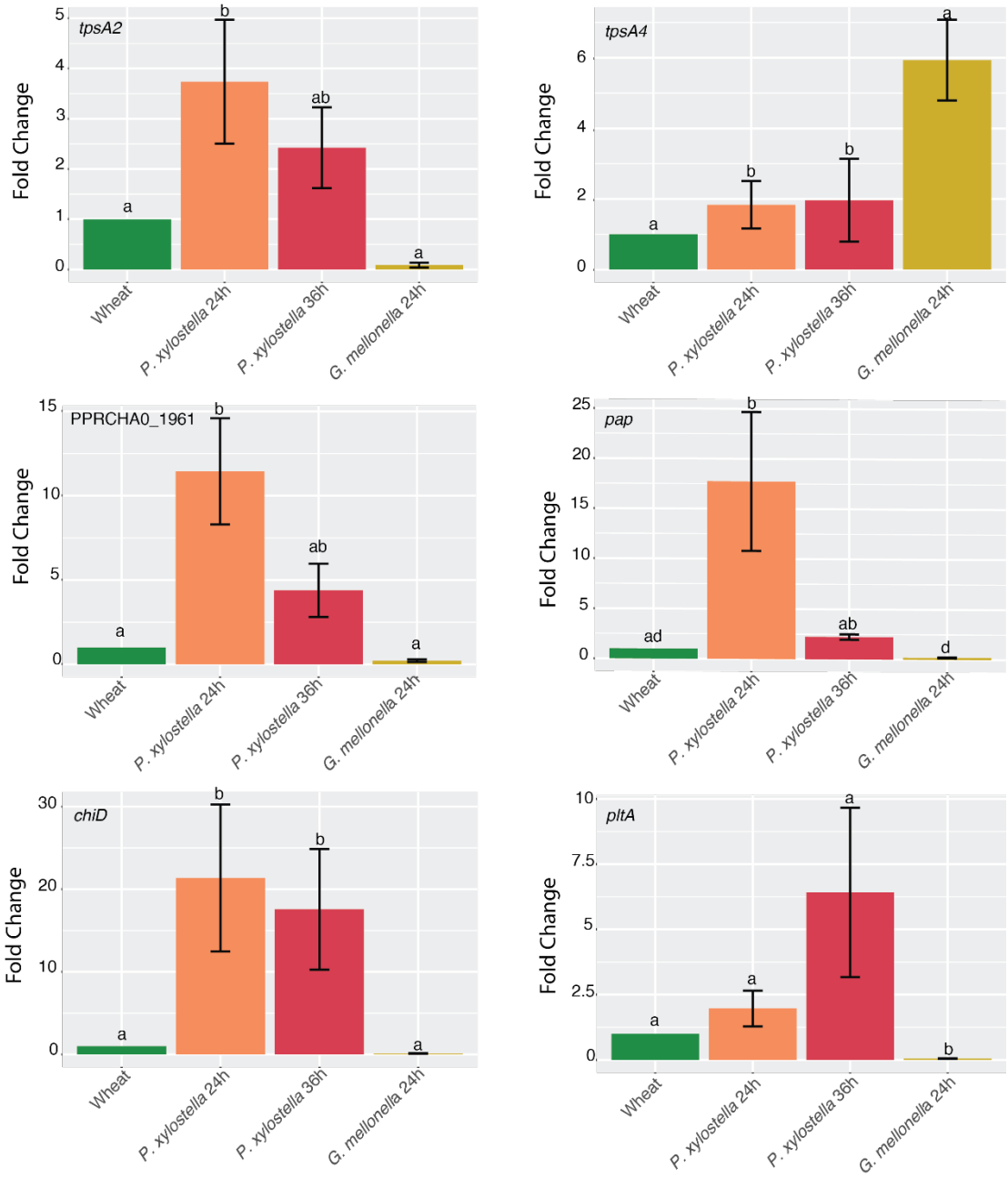


**Figure S3. Confirmation of RNA-sequencing results by qPCR.** Quantitative PCR was performed on *tpsA2*, *tpsA4*, transposase PPRCHA0_1961, *pap*, *chiD*, and *pltA* genes with the same samples as used for RNA-sequencing. The figure shows gene expression normalized to that on wheat roots (normalized gene expression on wheat roots = 1). Columns with different letters are statistically different at $P<0.05$ (Kruskal-Wallis analysis followed by Dunn's post hoc test in R).

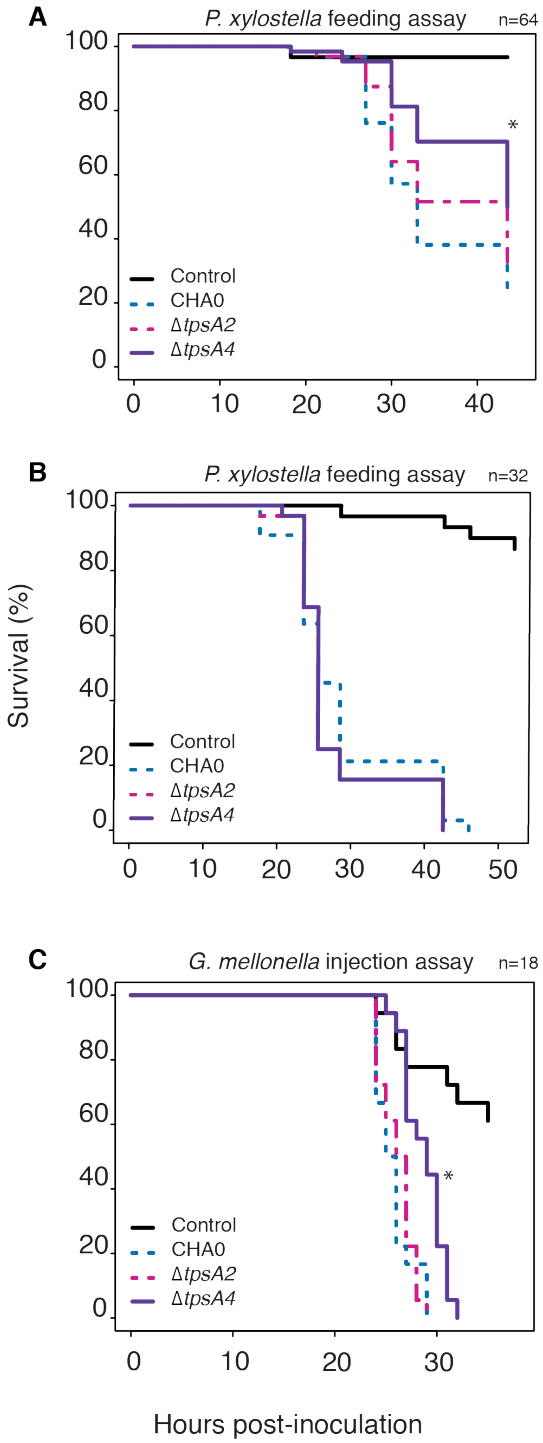


**Figure S4. Survival of *Plutella xylostella* and *Galleria mellonella* larvae when treated with Δ*tpsA2* and Δ*tpsA4* mutants of *P. protegens* CHA0.** A-B) Second instar *P. xylostella* larvae were exposed to artificial diet pellets spiked with 4·x $10^6$ cells of *tpsA2* or *tpsA4* deletion mutants. Thirty-two or sixty-four larvae were used per bacterial strain. C) Seventh instar *G. mellonella* larvae were injected with 2 x $10^3$ cells of *tpsA2* or *tpsA4* deletion mutants into the hemocoel. Eighteen larvae were used per bacterial strain. For all experiments, significant differences were assessed between bacterial treatments by log-rank test with a p-value<0.05.

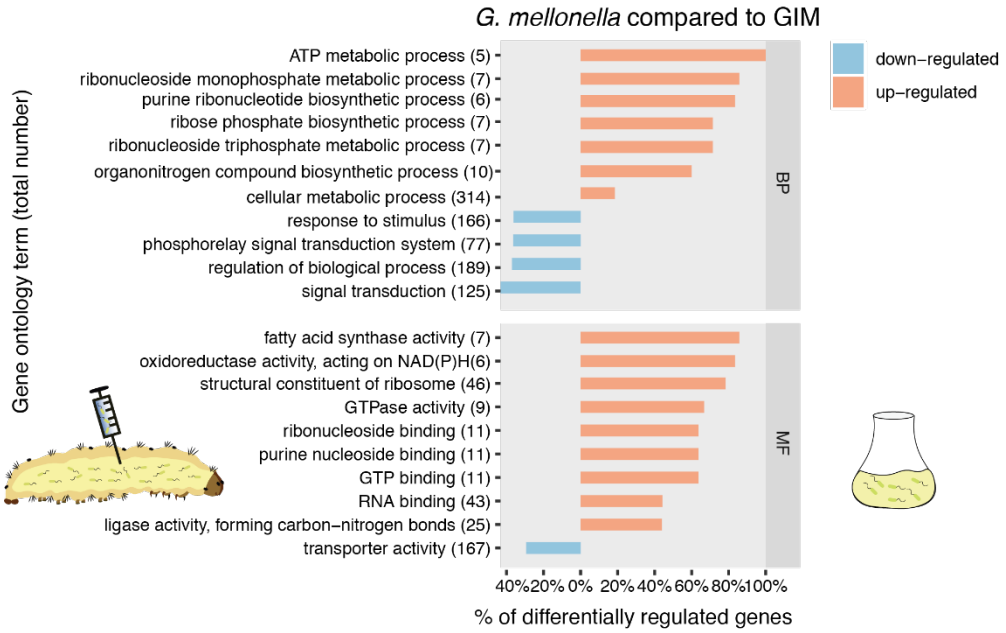


**Figure S5. Comparison of the transcriptomes of *P. protegens* CHA0 during colonization of the hemolymph of *Galleria mellonella* and Grace's insect medium.** CHA0 transcriptomes were compared using the general linear model pipeline of edgeR package in R. *G. mellonella* was compared to GIM as a reference. Total differentially expressed genes were subjected to a GO enrichment analysis. Significant GO terms for the given set of genes are shown. Total genes related to a GO term present in the CHA0 genome are given between brackets and the indicated percentage shows how many of those are differentially expressed in the comparison (p-value<0.001). *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection, GIM = Grace Insect medium.

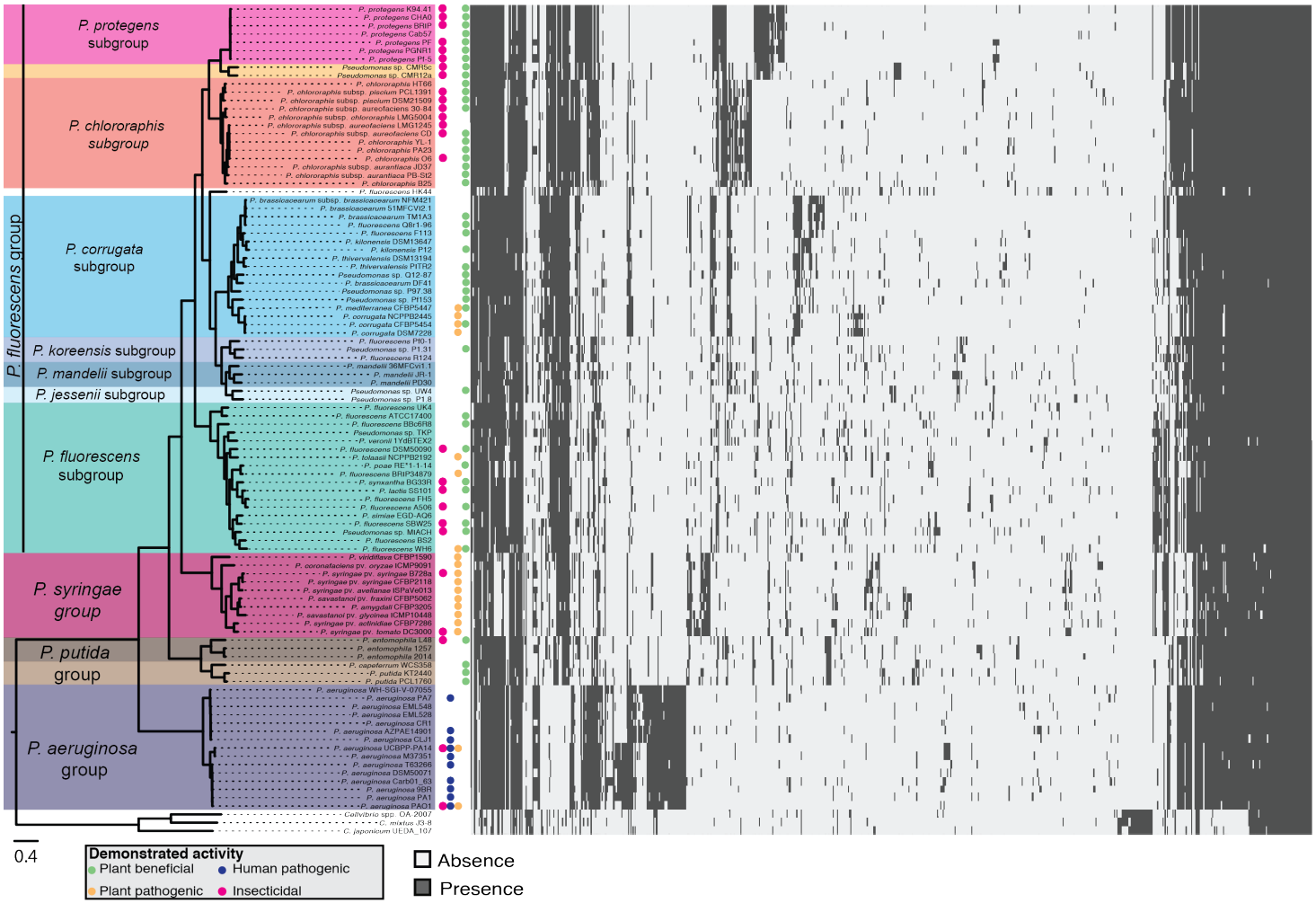


**Figure S6. Orthologue comparison based on whole proteomes between different strains of *Pseudomonas*.** Comparison of the full *in silico* proteomes of 97 pseudomonads belonging to phylogenetic groups harbouring insect pathogenic, human pathogenic, plant pathogenic and plant beneficial strains (groups and subgroups as defined by Hesse et al., [9]. Strain with described activity are marked in: pink for insecticidal activity (oral or injectable); dark-blue for human pathogenic activity; orange for plant pathogenic activity; green for plant-beneficial activity. Abbreviations: Am.: amidases; Enz.: enzymes

## 11.3. Supplementary Tables

**Supplementary Table S1. Reads obtained from Illumina NextSeq sequencing of RNA extracted from different CHA0 inoculated host/environments.** RNA 260/280 = absorbance ratio for RNA quality. Total reads= reads after trimming and quality check. Mapped= reads mapped against the *P. protegens* CHA0 genome; Counted= reads mapped to conding sequences. Wheat = wheat roots 1 week after inoculation, *P. xylostella* 24h/36h = *Plutella xylostella* 24 h and 36 h after oral infection; *G. mellonella* = *Galleria mellonella* hemolymph 24 h after hemocoel injection, LB = lysogeny broth; GIM = Grace's insect medium.

| Condition | Sample | RNA 260/280 | Reads | | | | |
|---|---|---|---|---|---|---|---|
| | | | Total | Mapped | % Mapped | Counted | % Counted |
| Wheat | 1 | 2.11 | 75 992 549 | 4 390 592 | 5.78 | 3 682 414 | 4.85 |
| Wheat | 2 | 2.05 | 68 841 535 | 7 606 894 | 11.05 | 6 403 568 | 9.30 |
| Wheat | 3 | 2.13 | 42 211 861 | 3 081 907 | 7.30 | 2 692 569 | 6.38 |
| Wheat | 4 | 2.09 | 42 066 805 | 2 459 093 | 5.85 | 2 108 168 | 5.01 |
| *Plutella*24h | 1 | 2.14 | 225 647 887 | 191 077 | 0.08 | 96 263 | 0.04 |
| *Plutella*24h | 2 | 2.15 | 291 918 440 | 209 593 | 0.07 | 134 556 | 0.05 |
| *Plutella*24h | 3 | 2.14 | 97 403 265 | 63 368 | 0.07 | 42 551 | 0.04 |
| *Plutella*24h | 4 | 2.16 | 120 197 312 | 142 690 | 0.12 | 91 670 | 0.08 |
| *Plutella*36h | 1 | 2.15 | 241 594 068 | 248 573 | 0.10 | 129 985 | 0.05 |
| *Plutella*36h | 2 | 2.14 | 21 2740 597 | 675 097 | 0.32 | 414 905 | 0.20 |
| *Plutella*36h | 3 | 2.16 | 127 029 836 | 500 219 | 0.39 | 264 853 | 0.21 |
| *Plutella*36h | 4 | 2.17 | 123 956 872 | 124 692 | 0.10 | 83 437 | 0.07 |
| *Galleria* | 1 | 2.15 | 135 529 889 | 21 861 431 | 16.13 | 10 635 551 | 7.85 |
| *Galleria* | 2 | 2.12 | 129 063 190 | 7 148 913 | 5.54 | 3 445 638 | 2.67 |
| *Galleria* | 3 | 2.1 | 48 399 955 | 8 630 776 | 17.83 | 3 918 036 | 8.10 |
| *Galleria* | 4 | 2.17 | 48 333 173 | 19 985 280 | 41.35 | 10 563 308 | 21.86 |
| LB | 1 | 2.13 | 61 202 272 | 60 356 041 | 98.62 | 27 122 810 | 44.32 |
| LB | 2 | 2.13 | 66 428 498 | 65 953 001 | 99.28 | 31 134 777 | 46.87 |
| LB | 3 | 2.13 | 63 401 498 | 62 486 186 | 98.56 | 35 990 967 | 56.77 |
| LB | 4 | 2.13 | 88 252 664 | 87 093 887 | 98.69 | 48 536 989 | 55.00 |
| GIM | 1 | 2.13 | 56 654 217 | 55 975 156 | 98.80 | 23 073 397 | 40.73 |
| GIM | 2 | 2.08 | 65 782 951 | 65 419 039 | 99.45 | 21 209 037 | 32.24 |
| GIM | 3 | 2.12 | 80 495 439 | 79 761 444 | 99.09 | 30 392 689 | 37.76 |
| GIM | 4 | 2.13 | 79 355 862 | 78 784 965 | 99.28 | 29 412 337 | 37.06 |

**Table S2. List of *Pseudomonas* strains used in the orthologue analysis. IP = Insect pathogen. PP = Plant Pathogen. HP = Human Pathogen. PB =Plant Beneficial.**

| Strain | Isolation | Activity | References | Assembly |
|---|---|---|---|---|
| *P. protegens* K94.41 | Cucumber root | IP, PB | [10] | GCF_001269485.1_ASM126948v1 |
| *P. protegens* CHA0 | Tobacco root | IP, PB | [11] | GCF_900560965.1_PPRCHA0 |
| *P. protegens* BRIP | Cyclops | IP, PB | [12] | GCF_001269495.1_ASM126949v1 |
| *P. protegens* Cab57 | Shepherd's purse | PB | [13] | GCF_000828695.1_ASM82869v1 |
| *P. protegens* PF | Wheat leaf | IP, PB | [14] | GCF_001269465.1_ASM126946v1 |
| *P. protegens* PGNR1 | Tobacco root | IP, PB | [15] | GCF_001269475.1_ASM126947v1 |
| *P. protegens* Pf-5 | soil | IP, PB | [16] | GCF_000012265.1_ASM1226v1 |
| *Pseudomonas* sp. CMR5c | Red cocoyan | IP, PB | [17, 18] | GCF_003850545.1_ASM385054v1 |
| *Pseudomonas* sp. CMR12a | Red cocoyan | IP, PB | [17, 18] | GCF_003850565.1_ASM385056v1 |
| *P. chlororaphis* HT66 | Rice root | PB | [19] | GCF_000597925.1_ASM59792v1 |
| *P. chlororaphis* subsp. piscium PCL1391 | Tomato root | IP, PB | [20] | GCF_003850445.1_ASM385044v1 |
| *P. chlororaphis* subsp. piscium DSM 21509 | European perch | IP, PB | [21] | GCF_003850345.1_ASM385034v1 |
| *P. chlororaphis* subsp. aureofaciens 30-84 | wheat seed | IP, PB | [22] | GCF_000281915.1_ASM28191v1 |
| *P. chlororaphis* subsp. chlororaphis LMG 5004 | Contaminated plate | IP | [23] | GCF_001269625.1_ASM126962v1 |
| *P. chlororaphis* subsp. aureofaciens LMG 1245 | River Clay | IP | [24] | GCF_001269575.1_ASM126957v1 |
| *P. chlororaphis* subsp. aureofaciens CD | Cyclops | IP, PB | [12, 17] | GCF_001269595.1_ASM126959v1 |
| *P. chlororaphis* YL-1 | Soybean root tip | PB | [25] | GCF_000512485.1_PCYL_1 |
| *P. chlororaphis* PA23 | Soybean | PB | [26, 27] | GCF_000698865.1_ASM69886v1 |
| *P. chlororaphis* O6 | Wheat root | IP, PB | [28] | GCF_000264555.1_ASM26455v1 |
| *P. chlororaphis* subsp. aurantiaca JD37 | Other | PB | [29] | GCF_000761195.1_ASM76119v1 |
| *P. chlororaphis* subsp. aurantiaca PB-St2 | Sugar cane | PB | [30] | GCF_000506385.1_PcsubspaPBSt2-2.0 |
| *P. chlororaphis* B25 | Plant | PB | [31] | GCF_003851985.1_ASM385198v1 |
| *P. fluorescens* HK44 | ingeenered | | [32] | GCF_000217955.2_PfluHK442.0 |
| *P. brassicacearum* subsp. brassicacearum NFM421 | Arabidopsis | | [33] | GCF_000194805.1_ASM19480v1 |
| *P. brassicacearum* 51MFCVI2.1 | Arabidopsis | | [34] | GCF_000510785.1_ASM51078v1 |
| *P. brassicacearum* TM1A3 | Tomato root | PB | [35] | GCF_001269635.1_ASM126963v1 |
| *P. fluorescens* Q8r1-96 | soil | PB | [36] | GCF_000263695.1_ASM26369v2 |
| *P. fluorescens* F113 | Sugar beet root | PB | [37] | GCF_000237065.1_ASM23706v1 |
| *P. kilonensis* DSM 13647 | soil | | [17, 38] | GCF_001269885.1_ASM126988v1 |
| *P. kilonensis* P12 | Tobacco root | PB | [17, 35] | GCF_001269725.1_ASM126972v1 |
| *P. thivervalensis* DSM 13194 | Rapeseed | | [17, 33] | GCF_001269655.1_ASM126965v1 |
| *P. thivervalensis* PITR2 | Wheat root | PB | [17, 35] | GCF_001269685.1_ASM126968v1 |
| *Pseudomonas* sp. Q12-87 | Wheat root | PB | [17, 35] | GCF_001269755.1_ASM126975v1 |
| *P. brassicacearum* DF41 | Canola | PB | [27] | GCF_000585995.1_ASM58599v1 |
| *Pseudomonas* sp. P97.38 | Cucumber root | PB | [17, 10] | GCF_001269745.1_ASM126974v1 |
| *Pseudomonas* sp. Pf153 | Cucumber root | PB | [17, 39] | GCF_001269775.1_ASM126977v1 |
| *P. mediterranea* CFBP 5447 | Tomato stem | PP, PB | [40, 41] | GCF_000774145.1_ASM77414v1 |

| | | | | |
|---|---|---|---|---|
| *P. corrugata* NCPPB2445 | Tomato stem | PP | [42, 43] | GCF_001411965.1_ASM141196v1 |
| *P. corrugata* CFBP 5454 | Tomato stem | PP, PB | [44, 45] | GCF_000522485.1_Pco1 |
| *P. corrugata* DSM 7228 | Tomato stem | PP | [17, 43] | GCF_001269905.1_ASM126990v1 |
| *P. fluorescens* Pf0-1 | Soil | | [46–48] | GCF_000012445.1_ASM1244v1 |
| *Pseudomonas* sp. P1.31 | Woodlouse | PB | [12, 17] | GCF_001269815.1_ASM126981v1 |
| *P. fluorescens* R124 | tepui | | [49] | GCF_000292795.1_PF-R124.01 |
| *P. mandelii* 36MFCvi1.1 | | | | GCF_000381285.1_ASM38128v1 |
| *P. mandelii* JR-1 | Water | | [50, 51] | GCF_000257545.3_ASM25754v3 |
| *P. mandelii* PD30 | soil | | [52, 53] | GCF_000690555.2_Pmandelii1.0 |
| *Pseudomonas* sp. UW4 | Reed root | PB | [54] | GCF_000316175.1_ASM31617v1 |
| *Pseudomonas* sp. P1.8 | Earthworm | | [12, 17] | GCF_001269805.1_ASM126980v1 |
| *P. fluorescens* UK4 | Water | | [55, 56] | GCF_000730425.1_ASM73042v1 |
| *P. fluorescens* ATCC 17400 | Hen egg | PB | [57] | GCF_000708695.2_ATCC_17400 |
| *P. fluorescens* BBc6R8 | fungus | PB | [58, 59] | GCF_000297195.2_Pseudomonas.strain_BBc6R8_v2.0 |
| *Pseudomonas* sp. TKP | HCH soil | | [60] | GCF_000508205.1_ASM50820v1 |
| *P. veronii* 1YdBTEX2 | Contaminated soil | | [61] | GCF_000350565.1_P.ver1YdBTEX2v.1 |
| *P. fluorescens* DSM 50090 | Prefilter tanks | IP, PB | [17, 62] | GCF_001269845.1_ASM126984v1 |
| *P. tolaasii* NCPPB 2192 | Mushrom | PP | [63, 64] | GCF_002813445.1_ASM281344v1 |
| *P. poae* RE*1.1.14 | Sugar Beet root | PB | [65, 66] | GCF_000336465.1_ASM33646v1 |
| *P. fluorescens* BRIP34879 | Barley glume | PP | [67] | GCF_000334015.1_BRIP34879v2.0 |
| *P. synxantha* BG33R | Peach tree root | IP, PB | [68, 69] | GCF_000263715.2_ASM26371v2 |
| *P. lactis* SS101 | Wheat root | IP, PB | [17, 70] | GCF_000263675.1_ASM26367v2 |
| *P. fluorescens* FH5 | Fresh water | | [71] | GCF_000511155.2_v1 |
| *P. fluorescens* A506 | Pear tree leaf | IP, PB | [69, 72, 73] | GCF_000262325.2_ASM26232v2 |
| *P. simiae* EGD-AQ6 | Sewage Sludge | | [74] | GCF_000465595.1_GS_De_Novo_Assembly |
| *P. fluorescens* SBW25 | Sugar beet leaves | IP, PB | [47, 75] | GCF_000009225.2_ASM922v1 |
| *Pseudomonas* sp. MIACH | Wheat root | IP, PB | [17, 76] | GCF_001269925.1_ASM126992v1 |
| *P. fluorescens* BS2 | Soil | | [77] | GCF_000308175.1_PseuFluoBS2 |
| *P. fluorescens* WH6 | Wheat root | PP, PB | [78, 79] | GCF_000166515.1_WH6_v1 |
| *P. viridiflava* CFBP 1590 | Diseased Cherry | PP | [80] | GCF_900184295.1_Chr_1 |
| *P. coronafaciens* pv. oryzae ICMP 9091 | rice leaf | PP | [81] | GCF_003701785.1_ASM370178v1 |
| *P. syringae* pv. syringae B728a | Bean leaf | IP, PP | [82–84] | GCF_000012245.1_ASM1224v1 |
| *P. syringae* pv. syringae CFBP2118 | Sweet Cherry | PP | [85] | GCF_900235865.1_CFBP2118 |
| *P. syringae* pv. avellanae str. ISPaVe013 | hazelnut | PP | [86] | GCF_000302795.1_Pav013_1.0 |
| *P. savastanoi* pv. fraxini CFBP 5062 | Ash | PP | [87] | GCF_001538155.1_ASM153815v1 |
| *P. amygdali* CFBP 3205 | Almond tree leaf | PP | [88, 89] | GCF_000935645.1_PSAVPseNe107-G1 |
| *P. savastanoi* pv. glycinea ICMP 10448 | Soybean | PP | [90] | GCF_003699735.1_ASM369973v1 |
| *P. syringae* pv. actinidiae CFBP 7286 | Kiwi fruit | PP | [91, 92] | GCF_000245415.1_ASM24541v1 |
| *P. syringae* pv. tomato str. DC3000 | Tomato | IP, PP | [82, 93, 94] | GCF_000007805.1_ASM780v1 |
| *P. entomophila* L48 | Drosophila | IP, PB | [95, 96] | GCF_000026105.1_ASM2610v1 |
| *P. entomophila* 1257 | Soil | | | GCF_003940825.1_ASM394082v1 |
| *P. entomophila* 2014 | Soil | | | GCF_003940785.1_ASM394078v1 |

| | | | | |
|---|---|---|---|---|
| *P. capeferrum* WCS358 | Potato root | PB | [64, 97, 98] | GCF_000731675.1_ASM73167v1 |
| *P. putida* KT2440 | Soil | PB | [96, 99–101] | GCF_000007565.2_ASM756v2 |
| *P. putida* PCL1760 | Avocado root | PB | [102] | GCF_001282125.1_ASM128212v1 |
| *P. aeruginosa* WH-SGI-V-07055 | Clinical isolate | | [103, 104] | GCF_001450355.1_WH-SGI-V-07055 |
| *P. aeruginosa* PA7 | non-respiratory human isolate | HP | [104–107] | GCF_000017205.1_ASM1720v1 |
| *P. aeruginosa* EML548 | unknown | | [104, 107, 108] | GCF_001280765.1_ASM128076v1 |
| *P. aeruginosa* EML528 | unknown | | [104, 107, 109] | GCF_001280755.1_ASM128075v1 |
| *P. aeruginosa* CR1 | Chili root | | [104] | GCF_003025345.2_ASM302534v2 |
| *P. aeruginosa* AZPAE14901 | Pus | HP | [110] | GCF_000791105.1_AZPAE14901 |
| *P. aeruginosa* CLJ1 | Hemorrhagic pneumonia | HP | [105, 106] | GCF_003032395.1_ASM303239v1 |
| *P. aeruginosa* UCBPP-PA14 | Human burn | IP, PP, HP | [104, 111–114] | GCF_000014625.1_ASM1462v1 |
| *P. aeruginosa* M37351 | Clinical isolate | HP | [104] | GCF_001516385.1_ASM151638v1 |
| *P. aeruginosa* T63266 | Clinical isolate | HP | [104] | GCF_001516105.1_ASM151610v1 |
| *P. aeruginosa* DSM 50071 | unknown | | [115] | GCF_001042925.1_G1273 |
| *P. aeruginosa* Carb01 63 | Hospital sink | HP | [116] | GCF_000981825.1_ASM98182v1 |
| *P. aeruginosa* 9BR | Cystic Fibrosis patient | HP | [104, 117] | GCF_000223925.1_ASM22392v2 |
| *P. aeruginosa* PA1 | Lung infection | HP | [104, 118] | GCF_000496605.2_ASM49660v2 |
| *P. aeruginosa* PAO1 | Wound | IP, PP, HP | [99, 106, 114, 119] | GCF_000006765.1_ASM676v1 |
| *Cellvibrio* spp. OA.2007 | Activated sludge | | [120] | GCF_000953825.1_ASM95382v1 |
| *C. mixtus* J3.8 | Giant snail | | [121] | GCF_002268635.1_ASM226863v1 |
| *C. japonicum* UEDA 107 | Soil | | [122] | GCF_000019225.1_ASM1922v1 |

**Supplementary Table S3. Primers used for RT-qPCR.**

| Gene | Sequence | Annealing T°C | bp | Reference |
|---|---|---|---|---|
| *chiD* | ATCATCCGTCTGGTGGAAACC | | | |
| | TGATGATGAAGTGCTTGCCCT | 60 °C | 154 | This study |
| *fitD* | GCAGAAGCTGTTCCTGGCC | | | |
| | CTGCCCACCATCATCGAGTG | 60 °C | 215 | This study |
| *tpsA1* | TGATCCTCAACAACGCCATCA | | | |
| | ATCTGACCGTTCTCAACCACC | 65 °C | 277 | This study |
| *tpsA2* | TACGCCAAGAAGCTCAACGT | | | |
| | CGTTGGCGTCGATCTGGATA | 65 °C | 235 | This study |
| *tpsA4* | GTCAACATCGTCGCGCCCAA | | | |
| | TGAGTTGCGAGGCATTGCGG | 65 °C | 208 | This study |
| *pap* | AAGAAACCTACGATGCCGAGG | | | |
| | CATTCGTTGAGCAGCTTGACC | 60 °C | 154 | This study |
| *pltA* | CGATTCACTCCTGGTTCGACA | | | |
| | TCGGAGTTGGTGTAGTTCTGC | 60 °C | 181 | This study |
| PPRCHA0_1961 | GTACGCCTTTATCAAGCTGCG | | | |
| | TAACCGCTGGGATGGACTTTC | 62 °C | 81 | This study |
| rRNA 16S | ACTTTAAGTTGGGAGGAAGGG | | | |
| | ACACAGGAAATTCCACCACCC | 60 °C | 251 | [123] |

**Supplementary Table S4. Quantitative PCR conditions for LightCycler480 (Roche, Switzerland)**

| | Phase | °C | Acquisition mode | Time | Ramp Rate (°C/s) | Acquisitions (per °C) |
|---|---|---|---|---|---|---|
| | Preincubation | 95 | | 15 min | | |
| Quant. | Amplification | 95 | none | 15 s | 4.4 | |
| | | * | none | 20 s | 2.2 | |
| | | 72 | Single/none** | 30 s | 4.4 | |
| | | ** | none | 5 s | 4.8 | |
| Melting | Melting | 95 | none | 5 s | 4.4 | |
| | Curve | 65 | none | 1 min | 2.2 | |
| | | 97 | continuous | - | 0.06 | 10 |
| | Cooling | 40 | none | 30 s | 2.2 | |

*Different temperatures from Supplementary Table S1 for the corresponding primers.

** To avoid false amplification due to primer dimer, amplification protocols had an extra step at 86°C for *tpsA2* and at 83°C for PPRCHA0_1961 and *pap*. In absence of primer dimers, this step was not included.

**Table S5. Plasmids and primers used to create *tpsA2* and *tpsA4* deletion mutants**

| Plasmid or primer | Relevant characteristics or sequence | Reference |
|---|---|---|
| Plasmids | | |
| pEMG | pSEVA212S; oriR6K, lacZα MCS flanked by two I-SceI sites; Kmr, Apr | [7] |
| pSW-2 | oriRK2, xylS, Pm::I-sceI; Gmr | [7] |
| Primers | | |
| *tpsA2*-1 | 5'-CGGAATTCACCGCATCACCGAAAGCCAGCT-3' - EcoRI | This study |
| *tpsA2*-2 | 5'-GGGGTACCTACGTCCATGTGCGAATCATCC-3' - KpnI | This study |
| *tpsA2*-3 | 5'-GGGGTACCGATGCCAATGGCAAGGACACTA-3' - KpnI | This study |
| *tpsA2*-4 | 5'-CGGGATCCCAGCACCAATACCTGACCTCAT-3' - BamHI | This study |
| *tpsA4*-1 | 5'-CGGAATTCACTCGCAGCAGGTACAGCGCAA-3' - EcoRI | This study |
| *tpsA4*-2 | 5'-GGGGTACCGCCCTGGGGTGAAAGGTTGAAT-3' - KpnI | This study |
| *tpsA4*-3 | 5'-GGGGTACCCTGTCCAAGTCACCGGTCAACC-3' – KpnI | This study |
| *tpsA4*-4 | 5'-CGGGATCCGTCCAACTTGGGAATGCAGATA-3 - BamHI | This study |

**Supplementary Table S6. Twenty most upregulated and downregulated genes in the comparison of two hosts.** Gene= gene name; ID= gene entry ID; LogFC = fold change in expression; logCPM= counts per million in log2; LR= likelihood ratio; FDR= false discovery rate; product= protein product.

***Plutella xylostella* compared to wheat root**

| Gene/ID | Length | logFC | logCPM | LR | PValue | FDR | Product |
|---|---|---|---|---|---|---|---|
| *Upregulated* | | | | | | | |
| PPRCHA0_1501 | 1182 | 10.80 | 8.98 | 29.43 | 6E-08 | 5E-07 | acetyl-CoA C-acetyltransferase family protein |
| *argE_1* | 1164 | 10.79 | 10.22 | 26.96 | 2E-07 | 2E-06 | acetylornithine deacetylase |
| *pvdT* | 1974 | 9.88 | 9.25 | 34.64 | 4E-09 | 4E-08 | efflux ABC transporter%2C ATP-binding/permease protein |
| PPRCHA0_1961 | 732 | 9.19 | 11.75 | 34.51 | 4E-09 | 5E-08 | transposase |
| PPRCHA0_2789 | 441 | 8.84 | 4.90 | 26.29 | 3E-07 | 2E-06 | putative general secretion pathway protein C |
| PPRCHA0_3729 | 945 | 8.79 | 4.98 | 27.43 | 2E-07 | 1E-06 | |
| PPRCHA0_4959 | 921 | 8.11 | 9.92 | 25.34 | 5E-07 | 3E-06 | |
| PPRCHA0_3765 | 3090 | 7.53 | 9.77 | 31.47 | 2E-08 | 2E-07 | |
| PPRCHA0_3370 | 1281 | 7.19 | 1.00 | 60.34 | 8E-15 | 3E-13 | |
| PPRCHA0_5765 | 1002 | 6.74 | 7.37 | 24.49 | 7E-07 | 5E-06 | alpha/beta hydrolase family protein |
| PPRCHA0_6083 | 363 | 6.70 | -0.71 | 38.04 | 7E-10 | 9E-09 | putative RNA polymerase-binding protein DksA |
| PPRCHA0_2057 | 1050 | 6.58 | 3.75 | 43.17 | 5E-11 | 8E-10 | putative protein ORF5 in retron EC67 |
| PPRCHA0_2046 | 444 | 6.33 | 1.36 | 34.10 | 5E-09 | 6E-08 | phage P2 LysB-like protein LysB |
| *pap* | 1515 | 6.23 | 8.89 | 27.44 | 2E-07 | 1E-06 | polyphosphate:AMP phosphotransferase |
| PPRCHA0_2485 | 402 | 6.21 | -0.97 | 18.76 | 1E-05 | 7E-05 | hypothetical protein |
| PPRCHA0_5413 | 792 | 6.10 | 9.59 | 23.44 | 1E-06 | 8E-06 | |
| PPRCHA0_3100 | 1125 | 6.01 | 3.93 | 59.01 | 2E-14 | 5E-13 | |
| PPRCHA0_3366 | 1164 | 5.89 | -1.49 | 37.64 | 9E-10 | 1E-08 | |
| PPRCHA0_0315 | 2100 | 5.82 | 5.90 | 124.50 | 7E-29 | 3E-26 | TonB-dependent outermembrane receptor |
| PPRCHA0_5656 | 888 | 5.68 | -0.45 | 56.17 | 7E-14 | 2E-12 | |
| *Downregulated* | | | | | | | |
| *rebB_2* | 315 | -7.22 | 10.69 | 78.65 | 7E-19 | 5E-17 | RebB protein |
| *rebB_1* | 315 | -6.87 | 9.32 | 90.54 | 2E-21 | 2E-19 | RebB protein |
| PPRCHA0_2601 | 405 | -6.34 | 2.74 | 53.88 | 2E-13 | 5E-12 | hypothetical protein |
| PPRCHA0_4090 | 117 | -6.23 | 4.66 | 100.04 | 1E-23 | 3E-21 | |
| PPRCHA0_0054 | 660 | -6.18 | 4.77 | 110.23 | 9E-26 | 2E-23 | dual specificity phosphatase%2C catalytic domain protein |
| PPRCHA0_0785 | 660 | -5.92 | 8.96 | 87.06 | 1E-20 | 1E-18 | hypothetical protein |
| PPRCHA0_4091 | 318 | -5.90 | 4.00 | 81.12 | 2E-19 | 2E-17 | |
| PPRCHA0_0146 | 510 | -5.77 | 7.36 | 76.50 | 2E-18 | 1E-16 | RNA polymerase sigma-70 factor%2C ECF family |
| PPRCHA0_2602 | 411 | -5.74 | 5.25 | 135.80 | 2E-31 | 2E-28 | putative lipoprotein |
| PPRCHA0_1517 | 663 | -5.53 | 4.22 | 112.51 | 3E-26 | 9E-24 | SAM dependent methyltransferase |
| PPRCHA0_4829 | 696 | -5.46 | 6.24 | 57.29 | 4E-14 | 1E-12 | |
| PPRCHA0_4926 | 669 | -5.28 | 8.13 | 84.81 | 3E-20 | 3E-18 | |
| PPRCHA0_2603 | 1017 | -5.10 | 6.12 | 81.16 | 2E-19 | 2E-17 | hypothetical protein |
| PPRCHA0_0615 | 468 | -5.09 | 8.27 | 84.79 | 3E-20 | 3E-18 | hypothetical protein |
| PPRCHA0_4831 | 867 | -5.07 | 6.85 | 79.59 | 5E-19 | 3E-17 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *cptA* | 1743 | -5.06 | 7.16 | 165.86 | 6E-38 | 1E-34 | phosphoethanolamine transferase CptA |
| *deaD* | 1674 | -5.05 | 10.42 | 169.40 | 1E-38 | 3E-35 | cold-shock DEAD box protein A |
| PPRCHA0_0184 | 228 | -5.01 | 5.41 | 61.59 | 4E-15 | 2E-13 | Reb-like protein |
| PPRCHA0_4751 | 756 | -4.97 | 4.71 | 99.92 | 2E-23 | 3E-21 | |
| PPRCHA0_4089 | 816 | -4.97 | 4.26 | 60.66 | 7E-15 | 2E-13 | |

***Galleria mellonella* compared to wheat root**

| Gene/ID | Length | logFC | logCPM | LR | PValue | FDR | Product |
|---|---|---|---|---|---|---|---|
| *Upregulated* | | | | | | | |
| *hasAp* | 618 | 10.73 | 11.59 | 99.18 | 2E-23 | 4E-21 | heme acquisition protein HasAp |
| PPRCHA0_4215 | 225 | 9.00 | 10.90 | 43.61 | 4E-11 | 9E-10 | |
| PPRCHA0_1501 | 1182 | 7.60 | 8.98 | 18.07 | 2E-05 | 1E-04 | acetyl-CoA C-acetyltransferase family protein |
| PPRCHA0_0222 | 579 | 7.11 | 6.13 | 206.45 | 8E-47 | 5E-43 | hypothetical protein |
| *fitD* | 9015 | 7.08 | 8.37 | 117.24 | 3E-27 | 1E-24 | cytotoxin FitD |
| *pvdT* | 1974 | 6.98 | 9.25 | 21.15 | 4E-06 | 3E-05 | efflux ABC transporter%2C ATP-binding/permease protein |
| *pvdE* | 1653 | 6.95 | 7.21 | 55.32 | 1E-13 | 4E-12 | pyoverdine ABC transporter%2C permease/ATP-binding protein PvdE |
| *pvdA* | 1338 | 6.89 | 9.50 | 44.05 | 3E-11 | 8E-10 | L-ornithine 5-monooxygenase PvdA |
| *fitA* | 2142 | 6.70 | 5.89 | 90.75 | 2E-21 | 2E-19 | type I secretion system ATPase FitA |
| *pvdF* | 864 | 6.61 | 7.78 | 68.45 | 1E-16 | 9E-15 | N(5)-hydroxyornithine transformylase PvdF |
| *fitB* | 1389 | 6.56 | 4.42 | 88.13 | 6E-21 | 7E-19 | type I secretion membrane fusion protein FitB |
| *hasF* | 1338 | 6.55 | 5.18 | 94.36 | 3E-22 | 4E-20 | type 1 secretion system outer membrane protein HasF |
| *pvdP* | 1629 | 6.35 | 6.67 | 55.58 | 9E-14 | 3E-12 | chromophore maturation protein PvdP |
| *fpvA* | 2469 | 6.35 | 9.08 | 45.52 | 2E-11 | 4E-10 | TonB-dependent outermembrane ferripyoverdine receptor FpvA |
| PPRCHA0_0220 | 672 | 6.30 | 6.87 | 121.82 | 3E-28 | 1E-25 | putative lipoprotein |
| *hasE* | 1350 | 6.29 | 4.42 | 113.72 | 2E-26 | 5E-24 | type 1 secretion system membrane fusion protein HasE |
| *pvdM* | 1374 | 6.21 | 5.95 | 57.31 | 4E-14 | 2E-12 | putative dipeptidase precursor |
| *pvdN* | 1314 | 6.20 | 5.48 | 54.16 | 2E-13 | 7E-12 | chromophore maturation protein PvdN |
| PPRCHA0_5091 | 2055 | 6.16 | 6.84 | 84.18 | 5E-20 | 5E-18 | |
| *Downregulated* | | | | | | | |
| PPRCHA0_4090 | 117 | -13.50 | 4.66 | 162.84 | 3E-37 | 3E-34 | |
| *rebB_2* | 315 | -9.96 | 10.69 | 65.46 | 6E-16 | 3E-14 | RebB protein |
| PPRCHA0_2601 | 405 | -9.79 | 2.74 | 101.84 | 6E-24 | 1E-21 | hypothetical protein |
| *rebB_1* | 315 | -9.61 | 9.32 | 80.34 | 3E-19 | 3E-17 | RebB protein |
| PPRCHA0_4094 | 468 | -9.32 | -0.11 | 40.07 | 2E-10 | 5E-09 | |
| PPRCHA0_4095 | 219 | -9.15 | -0.44 | 47.58 | 5E-12 | 1E-10 | |
| PPRCHA0_4091 | 318 | -9.03 | 4.00 | 139.09 | 4E-32 | 3E-29 | |
| PPRCHA0_0184 | 228 | -8.77 | 5.41 | 94.23 | 3E-22 | 4E-20 | hypothetical protein |
| PPRCHA0_0785 | 660 | -8.22 | 8.96 | 79.43 | 5E-19 | 5E-17 | hypothetical protein |
| PPRCHA0_2602 | 411 | -8.04 | 5.25 | 186.67 | 2E-42 | 3E-39 | putative lipoprotein |
| PPRCHA0_0267 | 909 | -8.00 | 5.08 | 58.16 | 2E-14 | 1E-12 | fatty acid desaturase family protein |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PPRCHA0_2256 | 765 | -7.98 | -0.97 | 24.22 | 9E-07 | 7E-06 | hypothetical protein |
| PPRCHA0_4092 | 582 | -7.57 | 2.53 | 114.79 | 9E-27 | 3E-24 | |
| PPRCHA0_4506 | 666 | -7.43 | 4.57 | 83.67 | 6E-20 | 6E-18 | |
| PPRCHA0_4926 | 669 | -7.41 | 8.13 | 84.79 | 3E-20 | 4E-18 | |
| PPRCHA0_0331 | 222 | -7.36 | 1.15 | 16.68 | 4E-05 | 3E-04 | putative formate dehydrogenase%2C delta subunit |
| PPRCHA0_2599 | 1167 | -7.12 | 0.72 | 59.04 | 2E-14 | 7E-13 | efflux transporter%2C RND family%2C MFP subunit |
| PPRCHA0_1829 | 327 | -7.08 | 5.84 | 58.62 | 2E-14 | 8E-13 | putative secreted protein |
| *acoX* | 1101 | -6.94 | -0.50 | 41.58 | 1E-10 | 2E-09 | acetoin catabolism protein |
| PPRCHA0_1828 | 381 | -6.94 | 6.44 | 50.39 | 1E-12 | 4E-11 | hypothetical protein |

***Plutella xylostella* compared to *Galleria mellonella***

| Gene/ID | Length | logFC | logCPM | LR | PValue | FDR | Product |
|---|---|---|---|---|---|---|---|
| *Upregulated* | | | | | | | |
| PPRCHA0_1433 | 1290 | 10.13 | -1.38 | 67.10 | 3E-16 | 1E-14 | pyridine nucleotide-disulfide oxidoreductase family protein |
| PPRCHA0_2477 | 615 | 9.23 | -0.31 | 37.62 | 9E-10 | 8E-09 | translocator protein%2C LysE family |
| PPRCHA0_1961 | 732 | 8.75 | 11.75 | 32.51 | 1E-08 | 9E-08 | transposase |
| PPRCHA0_4959 | 921 | 8.48 | 9.92 | 26.82 | 2E-07 | 1E-06 | |
| PPRCHA0_3171 | 1062 | 8.29 | -1.46 | 24.95 | 6E-07 | 3E-06 | |
| PPRCHA0_0267 | 909 | 8.20 | 5.08 | 62.13 | 3E-15 | 1E-13 | fatty acid desaturase family protein |
| PPRCHA0_1361 | 795 | 7.79 | 1.69 | 148.03 | 5E-34 | 1E-30 | Acyltransferase |
| *praB* | 507 | 7.78 | 6.84 | 56.13 | 7E-14 | 2E-12 | protein activator of alkane oxidation PraB |
| PPRCHA0_2256 | 765 | 7.61 | -0.97 | 15.90 | 7E-05 | 2E-04 | hypothetical protein |
| *acoX* | 1101 | 7.42 | -0.50 | 43.00 | 5E-11 | 7E-10 | acetoin catabolism protein |
| PPRCHA0_2696 | 1206 | 7.37 | -0.84 | 62.02 | 3E-15 | 1E-13 | CoA-transferase%2C family III |
| PPRCHA0_4090 | 117 | 7.27 | 4.66 | 13.57 | 2E-04 | 6E-04 | |
| PPRCHA0_1829 | 327 | 7.24 | 5.84 | 63.31 | 2E-15 | 6E-14 | putative secreted protein |
| PPRCHA0_4631 | 1320 | 7.20 | 10.33 | 32.42 | 1E-08 | 9E-08 | |
| PPRCHA0_2764 | 933 | 7.16 | -1.48 | 33.22 | 8E-09 | 6E-08 | putative triacylglycerol lipase |
| PPRCHA0_2965 | 1353 | 7.12 | 0.61 | 47.86 | 5E-12 | 8E-11 | oxidoreductase%2C short chain dehydrogenase/reductase family protein |
| *argE_1* | 1164 | 7.03 | 10.22 | 15.86 | 7E-05 | 2E-04 | acetylornithine deacetylase |
| PPRCHA0_1828 | 381 | 6.98 | 6.44 | 53.16 | 3E-13 | 7E-12 | hypothetical protein |
| *mdcD* | 852 | 6.95 | 1.20 | 37.11 | 1E-09 | 1E-08 | malonate decarboxylase%2C beta subunit |
| PPRCHA0_0779 | 1467 | 6.87 | 3.55 | 149.00 | 3E-34 | 9E-31 | N-acyl-D-aspartate deacylase |
| *Downregulated* | | | | | | | |
| *hasAp* | 618 | -8.31 | 11.59 | 133.16 | 8E-31 | 1E-27 | heme acquisition protein HasAp |
| PPRCHA0_4215 | 225 | -5.64 | 10.90 | 41.45 | 1E-10 | 1E-09 | |
| *pvdA* | 1338 | -5.38 | 9.50 | 54.42 | 2E-13 | 4E-12 | L-ornithine 5-monooxygenase PvdA |
| PPRCHA0_0222 | 579 | -5.30 | 6.13 | 168.95 | 1E-38 | 8E-35 | hypothetical protein |
| PPRCHA0_4278 | 4959 | -5.08 | 8.18 | 84.17 | 5E-20 | 6E-18 | |
| PPRCHA0_0221 | 186 | -5.05 | 6.95 | 93.14 | 5E-22 | 9E-20 | hypothetical protein |
| *rpsN* | 306 | -4.98 | 11.69 | 85.36 | 2E-20 | 3E-18 | ribosomal protein S14 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *pvdF* | 864 | -4.94 | 7.78 | 73.73 | 9E-18 | 6E-16 | N(5)-hydroxyornithine transformylase PvdF |
| PPRCHA0_0220 | 672 | -4.92 | 6.87 | 125.03 | 5E-29 | 5E-26 | putative lipoprotein |
| *rplI* | 447 | -4.88 | 11.01 | 117.86 | 2E-27 | 1E-24 | ribosomal protein L9 |
| *fpvA* | 2469 | -4.69 | 9.08 | 50.11 | 1E-12 | 3E-11 | TonB-dependent outermembrane ferripyoverdine receptor FpvA |
| *pvdM* | 1374 | -4.67 | 5.95 | 53.70 | 2E-13 | 5E-12 | putative dipeptidase precursor |
| PPRCHA0_3207 | 381 | -4.63 | 5.95 | 68.40 | 1E-16 | 6E-15 | |
| *pth* | 585 | -4.58 | 8.18 | 96.35 | 1E-22 | 2E-20 | aminoacyl-tRNA hydrolase |
| *pvdH* | 1413 | -4.56 | 6.90 | 59.49 | 1E-14 | 4E-13 | 2%2C4-diaminobutyrate 4-transaminase |
| *fumC_1* | 1377 | -4.49 | 8.96 | 93.65 | 4E-22 | 8E-20 | fumarate hydratase%2C class II |
| *pchD* | 1668 | -4.48 | 5.68 | 60.99 | 6E-15 | 2E-13 | salicyl-AMP ligase |
| PPRCHA0_5088 | 600 | -4.46 | 11.73 | 81.96 | 1E-19 | 1E-17 | |
| PPRCHA0_3516 | 882 | -4.45 | 3.79 | 47.71 | 5E-12 | 8E-11 | |
| PPRCHA0_0236 | 660 | -4.44 | 5.09 | 28.58 | 9E-08 | 5E-07 | amino acid ABC transporter%2C permease protein%2C 3-TM region%2C His/Glu/Gln/Arg/opine family |

***Galleria mellonella*** **compared to GIM**

| Gene/ID | Length | logFC | logCPM | LR | PValue | FDR | Product |
|---|---|---|---|---|---|---|---|
| *Upregulated* | | | | | | | |
| *acoB* | 1014 | 7.64 | 3.21 | 66.80 | 3E-16 | 1E-14 | acetoin dehydrogenase E1 component%2C beta subunit |
| PPRCHA0_1501 | 1182 | 7.43 | 8.98 | 18.01 | 2E-05 | 9E-05 | acetyl-CoA C-acetyltransferase family protein |
| PPRCHA0_0220 | 672 | 6.15 | 6.87 | 122.02 | 2E-28 | 2E-25 | putative lipoprotein |
| *rplJ* | 501 | 6.14 | 12.60 | 56.96 | 4E-14 | 1E-12 | ribosomal protein L10 |
| *rplI* | 447 | 6.07 | 11.01 | 93.22 | 5E-22 | 8E-20 | ribosomal protein L9 |
| *rpmC* | 192 | 6.01 | 10.65 | 59.01 | 2E-14 | 4E-13 | ribosomal protein L29 |
| *rpsN* | 306 | 5.97 | 11.69 | 63.13 | 2E-15 | 6E-14 | ribosomal protein S14 |
| *puuA1* | 1377 | 5.96 | 5.80 | 94.05 | 3E-22 | 5E-20 | putative gamma-glutamylputrescine synthetase PuuA |
| *kgtP* | 1323 | 5.95 | 5.82 | 192.00 | 1E-43 | 7E-40 | alpha-ketoglutarate MFS transporter KgtP |
| *pth* | 585 | 5.88 | 8.18 | 87.59 | 8E-21 | 1E-18 | aminoacyl-tRNA hydrolase |
| PPRCHA0_5496 | 366 | 5.84 | 12.99 | 44.82 | 2E-11 | 3E-10 | |
| *rplE* | 540 | 5.83 | 13.18 | 47.95 | 4E-12 | 7E-11 | ribosomal protein L5 |
| *rpmD* | 177 | 5.75 | 12.00 | 54.86 | 1E-13 | 3E-12 | ribosomal protein L30 |
| *rplP* | 414 | 5.71 | 12.09 | 53.47 | 3E-13 | 5E-12 | ribosomal protein L16 |
| *rplX* | 315 | 5.71 | 11.73 | 55.28 | 1E-13 | 2E-12 | ribosomal protein L24 |
| *rpsQ* | 267 | 5.66 | 11.87 | 47.39 | 6E-12 | 8E-11 | 30S ribosomal protein S17 |
| *rplO* | 435 | 5.66 | 12.04 | 73.87 | 8E-18 | 5E-16 | ribosomal protein L15 |
| *rplN* | 369 | 5.65 | 12.11 | 62.67 | 2E-15 | 7E-14 | ribosomal protein L14 |
| PPRCHA0_5088 | 600 | 5.64 | 11.73 | 67.15 | 3E-16 | 1E-14 | |
| *rpsD* | 621 | 5.46 | 12.34 | 66.93 | 3E-16 | 1E-14 | ribosomal protein S4 |
| *Downregulated* | | | | | | | |
| PPRCHA0_1454 | 237 | -9.84 | 10.00 | 68.79 | 1E-16 | 5E-15 | putative membrane protein |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *rebB_2* | 315 | -9.14 | 10.69 | 58.76 | 2E-14 | 5E-13 | RebB protein |
| PPRCHA0_3353 | 351 | -9.01 | 10.22 | 87.10 | 1E-20 | 1E-18 | |
| *rebB_1* | 315 | -8.91 | 9.32 | 72.82 | 1E-17 | 8E-16 | RebB protein |
| PPRCHA0_0706 | 207 | -8.80 | 10.61 | 96.26 | 1E-22 | 2E-20 | Flp/Fap pilin component family protein |
| PPRCHA0_4572 | 1059 | -8.43 | 8.55 | 74.09 | 7E-18 | 5E-16 | |
| *arcB* | 1062 | -8.35 | 6.63 | 50.09 | 1E-12 | 2E-11 | ornithine cyclodeaminase |
| PPRCHA0_2341 | 1545 | -8.33 | 8.90 | 110.62 | 7E-26 | 3E-23 | TROVE domain protein |
| PPRCHA0_2182 | 186 | -8.20 | 7.27 | 116.50 | 4E-27 | 3E-24 | hypothetical protein |
| PPRCHA0_0154 | 246 | -8.09 | 6.27 | 80.16 | 3E-19 | 3E-17 | hypothetical protein |
| PPRCHA0_0331 | 222 | -8.07 | 1.15 | 22.64 | 2E-06 | 1E-05 | putative formate dehydrogenase%2C delta subunit |
| *cysI_1* | 1674 | -7.91 | 7.58 | 143.44 | 5E-33 | 7E-30 | sulfite reductase (NADPH) hemoprotein%2C beta-component |
| PPRCHA0_2896 | 234 | -7.83 | 4.14 | 76.14 | 3E-18 | 2E-16 | hypothetical protein |
| PPRCHA0_4299 | 1872 | -7.70 | 9.36 | 89.80 | 3E-21 | 4E-19 | |
| PPRCHA0_1828 | 381 | -7.67 | 6.44 | 58.35 | 2E-14 | 6E-13 | hypothetical protein |
| PPRCHA0_1829 | 327 | -7.61 | 5.84 | 65.61 | 6E-16 | 2E-14 | putative secreted protein |
| PPRCHA0_3455 | 138 | -7.23 | 10.76 | 72.43 | 2E-17 | 1E-15 | |
| PPRCHA0_0785 | 660 | -7.23 | 8.96 | 66.70 | 3E-16 | 1E-14 | hypothetical protein |
| PPRCHA0_4926 | 669 | -7.16 | 8.13 | 80.71 | 3E-19 | 2E-17 | |
| *aphA_1* | 1044 | -7.16 | 6.95 | 45.44 | 2E-11 | 2E-10 | acetylpolyamine aminohydrolase |

**Supplementary Table S7. Similarities of the predicted two-partner secretion A (TpsA) – like proteins of *P. protegens* CHA0 with related proteins in pathogenic bacteria.**

| | | Protein in *P. protegens* CHA0 (gen ID) | | | |
|---|---|---|---|---|---|
| **Organism** | **Protein Homologue (gen ID)** | **TpsA1** (PPRCHA0_0169) | **TpsA2** (PPRCHA0_0626) | **TpsA3** (PPRCHA0_1575) | **TpsA4** (PPRCHA0_4278) |
| *Pseudomonas aeruginosa* PA7 | ExlA (PSPA7_4642) | 44.35% | 39.35% | 43.85% | 59.53% |
| *Serratia. marcescens* | ShlA (A8A12_12190) | 33.69% | 28.82% | 31.85% | 35.34% |
| *Proteus mirabilis* | HmpA (F4W58_04730) | 32.46% | 31.58% | 29.66% | 31.22% |
| *Photorhabdus. luminescens* | PhlA (TP56_RS21180) | 33.64% | 31.75% | 29.07% | 32.19% |
| *Bordetella pertussis* | FhaB (L565_RS14220) | 38.27% | 28.04% | 37.23% | 37.23% |
| *Haemophilus. influenza* | HMW1A (ADC26_RS06775) | 29.21% | 27.42% | 29.31% | 26.29% |

## 11.4. RNAseq analysis script

```
#Title paper:Transcriptome plasticity underlying plant root
colonization and insect invasion by Pseudomonas protegens
#Script author: Pilar Vesga & Daniel Croll
#Date: November 2019
#
##########################################

#Adaptor and Quality trimming
#Program: Trimmomatic 0-2.36
trimmomatic SE -threads 4 -phred33 -trimlog Sample_Log.txt
Sample.fastq.gz SampleTrimmed.fastq.gz ILLUMINACLIP:TruSeq3-
SE.fa:2:30:10 SLIDINGWINDOW:4:20 MINLEN:30

#Quality check
#Progam: FastQC 0.11.5

#Mapping against Pseudomonas protegens CHA0 (PPRCHA0) reference
genome with accesion number LS999205.1
#Program: STAR 2.3.5a

  #Generation of genome index
  STAR --runThreadN 10 --runMode genomeGenerate --genomeDir
genomeindex/ --genomeFastaFiles genomeindex/PPRCHA0.fas --
genomeSAindexNbases 10

  #Alignment
  STAR --runThreadN 10 --outFilterMismatchNmax 20 --
outFilterMultimapNmax 5 --genomeDir genomeindex/ --readFilesCommand
zcat --readFilesIn SampleTrimmed.fastq.gz --genomeLoad LoadAndKeep
--outFileNamePrefix Sample

#Data format manipulation
#Program: samtools 1.2
  #transform sam into bam, order and index the file.
  samtools view -bS -@ 24 SampleAligned.out.sam > Sample.bam
  #Sort Alignments
  samtools sort -@ 24 Sample.bam SampleSorted
  #Index Alignment entries
  samtools index SampleSorted.bam


#CDS mapped reads count
#Program: FeatureCounts 1.5.3
featureCounts -T 5 -Q 20 -s 2 -t CDS -g gene_name -O --verbose -f -
-fraction -a genomeindex/PPRCHA0.gtf -o FeatureCounts/Counts.txt
STAR_samtools/*Sorted.bam


#Cluster K-means, General Linear Model Analysis, GO enrichment and
Heatmaps analysis from RNA-seq data.
#Program: R 3.6.0

   #data
```

```
      metadata<- read.table("Metadata.csv", header=TRUE, sep=",")
#Library names and organization

      mytable<-read.table("Counts.csv", skip=1, header=TRUE,
sep=",")
      head(mytable)

      #separate only the counts from the rest of the info
      mycounts<-mytable[,c(7:30)] #change according to the sample
      head(mycounts)

      #name the rows
      row.names(mycounts)<-mytable[,1]
      head(mycounts)

   #Counts normalization
   #tutorial: https://www.nature.com/articles/nprot.2013.099
   #Package: edgeR 3.26.6
      #Normalization and counts per million reads. Normalizes
regarding the size of the library
      cpm_y=cpm(mycounts, normalized.lib.sizes=TRUE)
      head (cpm_y)

      #remove data with low counts
      keep=rowSums(cpm_y > 1)>=4 #we create a list of the genes
with more than 1 count in the 4 biological replicates we have.
      mycounts.filtered=mycounts[keep,] #we keep only the genes
within the keep list.
      mytable.filtered<-mytable[keep,]

      #Make a Differential Gene Expression List
      y=DGEList(counts=mycounts.filtered,
group=metadata[,"Condition"],
genes=mytable.filtered[,c("Geneid","Length") ])
      y=calcNormFactors(y,method="TMM") #Normalization with TMM
method.

      #Multidimensaional scaling plot (MDS). Differences between
the highest 2-fold change between each pair of samples
      par(mar=c(5,4,4,2))
      plotMDS(y,labels=metadata[,"LibraryName"],
col=c("gold3","#4292c6", "#253494", "#fc8d59","#d53e4f",
"#238b45")[factor(metadata$Condition)])

      #Mean variance relationship of each gene
      d=estimateCommonDisp(y)
      d=estimateTagwiseDisp(d)



  #Cluster Analysis by K-means
  #Tutorial: https://2-bitbio.com/2017/10/clustering-rnaseq-data-
using-k-means.html
  library(edgeR)
  library(ggplot)
  library(dplyr)
  library(reshape)
```

```
  library(tidyR)
      #Prepare data. We have to summarise it by Condition/kind of
biological sample
      z<-cpm(y, normalized.lib.size=TRUE)
      z.m <- melt(z) #transforms the heatdata in just a 2 column
format with all the data in these two columns
      names(z.m) <- c("gene", "LibraryName", "counts")
      z.meta <- merge(z.m , metadata, by = "LibraryName", all.x =
T)
      z.sum <- z.meta %>% group_by(Condition, gene) %>% #group the
data by the Condition(kind of sample) per each gene
       summarise(mean_counts = mean(counts), sd=sd(counts),
N=length(counts), se = sd / sqrt(N)) %>% #takes this grouped data
and calculates the mean of each
       as.data.frame()
      z.sum.mat <- dcast(z.sum, gene ~ Condition)
      rownames(z.sum.mat)<-z.sum.mat[, "gene"]
      z.sum.mat<-z.sum.mat[, colnames(z.sum.mat) != "gene"]

      #we need to scale the data. This is so that we can identify
clusters of genes that share similar expression profiles rather
than similar expression levels.
      scaledata <- t(scale(t(z.sum.mat))) # Centers and scales
data.

      #How many clusters? (number K)
        #SSE method. The elbow sugests a suitable number of
clusters. Most realiable method.
        wss <- (nrow(scaledata)-1)*sum(apply(scaledata,2,var))
        for (i in 2:20) wss[i] <- sum(kmeans(scaledata,
                             centers=i)$withinss)
        plot(1:20, wss, type="b", xlab="Number of Clusters",
           ylab="Within groups sum of squares")

      #Clustering analysis.
      set.seed(2)
      kClust <- kmeans(scaledata, centers=6, nstart = 1000,
iter.max = 100) #initial analysis nstart=1000 iter=100
      #iter.max: max number of iterations centers=K, the number of
clusters
      kClusters <- kClust$cluster

      # function to find centroid in cluster i
      clust.centroid = function(i, dat, clusters) {
       ind = (clusters == i)
       colMeans(dat[ind,])
      }
      kClustcentroids <- sapply(levels(factor(kClusters)),
clust.centroid, scaledata, kClusters)

      #get in long form for plotting
      Kmolten <- melt(kClustcentroids)
      colnames(Kmolten) <- c('sample','cluster','value')


  #Differential Gene Expression: General Linear Model analysis
  #tutorial: https://www.nature.com/articles/nprot.2013.099
```

```
      metadata.glm<-read.csv("MetadataSample.csv", header = T)

      #build a design matrix to classify the samples according to
the conditions we want to compare. Following a single example of
Plutella vs Wheat
      design<-model.matrix(~0+Sample, data=metadata.glm) #Compare
the Plutella against the wheat
       colnames(design)<-c("Galleria","GM","Plutella","LB","Wheat")
       rownames(design) <- levels(metadata.glm$LibraryName)

      #estimate dispersion according to the linear model.
       g=estimateGLMTrendedDisp(y, design)
       g=estimateGLMTagwiseDisp(g, design)
       plotBCV(g)

      #Adjust each feature to a general linear model related to the
desing matrix. Alternative to the "exact test in the pair-wise
comparison".
      f=glmFit(g, design, robust=TRUE)

      #make the constrast variable to indicate what you want to
compare. The First one is compared to the second one (Plutella exp
over Wheat)
      con<-makeContrasts(PlutellavsWheat = Plutella - Wheat,
levels=design)

      #Likelihood ratio test specifying which column do we want as
a reference. In the nature protocols paper this would be the "de"
list
      PlutellavsWheat=glmLRT(f, contrast= con)
      summary(decideTests(PlutellavsWheat))
      plotMD(PlutellavsWheat)

      #Select how many hints you want in your table (change
nrow(de) by a value)
      tt_total_PlutellavsWheat<-topTags(PlutellavsWheat,
n=nrow(PlutellavsWheat))
       tt_total_PlutellavsWheat<-
merge(tt_total_PlutellavsWheat$table,
AnnotationCHA0[,c("Geneid","ID", "Product","PFAM")], by="Geneid")


  #Gente Ontology Enrichment for GLM. Same pipeline followed for K-
means analysis.
  library(edgeR)
  library(seqinr)
  library(reshape2)
  library(dplyr)
  library(tidyr)
  library(GOSim)
  library(GO.db)
  library(annotate)
  library(GSEABase)
  library(GOstats)
  library(data.table)
  library(GenomicFeatures)
```

```
  library(ggplot2)
  library(scales)

      #Generate the Gene Ontology Database for P. protegens CHA0

      AnnotationCHA0.Interpro <-
read.csv("PPRCHA0.protein.fa.summary.csv", sep=",")
      names(AnnotationCHA0.Interpro) <- c("ID", "SignalIP", "TMHMM",
"Pfam", "GOterms", "IPR_description", "Pathway", "CDD",
"ProSiteProfiles",
              "SMART", "SUPERFAMILY", "TIGRFAM", "Hamap")

            ### summarize per gene using | separator
            GOterms.df <- as.data.frame(AnnotationCHA0.Interpro %>%
group_by(ID) %>% filter(!GOterms =="")
                                    %>% distinct(ID, GOterms) %>%
summarize(GOterms = paste(GOterms, collapse = "|")))

            ### list all GOterms individually per gene
            GOlist <- GOterms.df %>% mutate(GOterms =
strsplit(as.character(GOterms), "\\,")) %>% unnest(GOterms)

            names(GOlist) <- c("ID","GoTerm")

            GOIds <- as.character(GOlist$GoTerm)
            evi <- rep("IEA",nrow(GOlist)) ####?
            genes <- as.character(GOlist$ID)

            frameData = data.frame(cbind(GOIds,evi,genes))

            frame=GOFrame(frameData,organism="CHA0")
            allFrame=GOAllFrame(frame)

            # define the GO database for later reference
            CHA0.GO.db <- allFrame@data

             # define universe and Param object
            universe.CHA0 <- as.character(unique(GOlist$ID))
            gsc.CHA0 <- GeneSetCollection(allFrame, setType =
GOCollection())



      comparisons=list(PlutellavsWheat=tt_total_PlutellavsWheat)



       for (i in names(comparisons)) {

        print(names(comparisons[i]))
        name<-names(comparisons[i])

        tt.df <- comparisons[[i]] #double bracket to save the
object as a data frame and not as a list

        #toptags
        tt.df.toptags<-tt.df[order(tt.df$PValue),]
```

```
        tt.df.toptags<-tt.df.toptags[1:10, ]
        write.csv(tt.df.toptags, paste0(name,".top20Tags.csv"),
row.names=F)

        FDRlim<-0.001
        logFClim<-1.5
        tt.up.df<- tt.df[tt.df$logFC >= logFClim & tt.df$FDR <=
FDRlim,]
        tt.down.df<- tt.df[tt.df$logFC <= -logFClim & tt.df$FDR <=
FDRlim,]

        write.csv(rbind(tt.up.df, tt.down.df), paste0(name,".up-
down.csv"), row.names=F)

        GO.df <- data.frame(matrix(nrow=0, ncol=9))
        Selection<-data.frame(matrix(nrow=0, ncol=0))

         for (dir in c("up-regulated","down-regulated")) {
          if (dir == "up-regulated") {
           gene.set <- as.character(tt.up.df$ID)
           print(dir)
          }

          if (dir == "down-regulated") {
           gene.set <- as.character(tt.down.df$ID)
           print(dir)
          }

         print(head(gene.set))


         # GO enrichment set general cutoffs
         p.cutoff <- 0.001
         min.term.size <- 5

          for (ont in c("MF", "BP")) {
           print(ont)

            hGtest.params <- GSEAGOHyperGParams(name="annotation",
geneSetCollection = gsc.CHA0, #GSEAHyper... not in the manual ???
                              geneIds = gene.set, universeGeneIds =
universe.CHA0,
                              ontology = ont, pvalueCutoff =
p.cutoff, conditional = TRUE, testDirection = "over")
            #normal warning saying that is removing the geneIDs that
are not in our geneset

            hGtest <- hyperGTest(hGtest.params)
            hGtest.summary <- summary(hGtest, pvalue = p.cutoff)
            print(hGtest.summary)
            names(hGtest.summary) <- c("GO Term", "Enrichment p-
value", "Odds Ratio", "Expected GO term count", "Effective GO term
count", "Total genes per GO", "Term")

            # trim by term count
```

```
          hGtest.trimmed <- hGtest.summary[hGtest.summary$"Total
genes per GO" >= min.term.size & hGtest.summary$"Enrichment p-
value" < p.cutoff,]


          if (nrow(hGtest.trimmed) > 0) {
           hGtest.trimmed$direction <- dir
           hGtest.trimmed$GOcategory <- ont
           GO.df <- plyr::rbind.fill(GO.df, hGtest.trimmed)

           #Extract the exact GO terms with the genes that belong
to it
           GO.significant<-as.data.frame(GO.df$`GO Term`)
           GO.significant$Term<-GO.df$Term
           colnames(GO.significant)<-c("go_id","Term")
           Geneids<-as.data.frame(geneIds(hGtest))
           colnames(Geneids)<-c("gene_id")
           Geneids <- merge(Geneids, CHA0.GO.db[c("go_id",
"gene_id")], by="gene_id")
           Select<- merge(GO.significant,
Geneids[c("gene_id","go_id")], by="go_id")
           Select<-dcast(Select, gene_id ~ Term)
           rownames(Select)<-Select[,1]
           Select[,"gene_id"]<-NULL
           rownames(Select) <- c()
           Selection <- plyr::rbind.fill(Selection, Select)

           } else { print("no data")}
         }

        write.csv(GO.df, file=paste0(name,".GOenrichment.csv"),
row.names=F)
        write.csv(Selection,
file=paste0(name,".GOenrichment_significantTerms.csv"),
row.names=F)

        }
       }



  #Heatmaps
  library(viridis)
  library(heatmap.plus)
  library(gplots)
  library(RColorBrewer)
  library(dplyr)
  library(reshape2)

       #calculates the counts per million
       tmm_counts <- cpm(d)

       #Calculate the average per gene per each kind of sample.
Transformation of the data fram
       heatdata.m <- melt(tmm_counts) #transforms the heatdata in
just a 2 column format with all the data in these two columns
       names(heatdata.m) <- c("gene", "LibraryName", "counts")
```

```
       heatdata.meta <- merge(heatdata.m, metadata, by =
"LibraryName", all.x = T)#merge the counts with the metadata info
for making the average
       heatdata.meta.means <- heatdata.meta %>% group_by(Condition,
gene) %>% #group the data by the Condition(kind of sample) per each
gene
        summarise(mean_counts = mean(counts)) %>% #takes this
grouped data and calculates the mean of each
        as.data.frame() #transform the data back into a data frame
       heatdata.meta.means.mat <- dcast(heatdata.meta.means, gene ~
Condition) #Undone melt to get the original matrix but with the
merged by sample
       heatdata.meta.means.mat.short <- heatdata.meta.means.mat[,
2:ncol(heatdata.meta.means.mat)] #takes only the columns from the
samples
       rownames(heatdata.meta.means.mat.short)<-
heatdata.meta.means.mat$gene #includes the row names of the genes.

       heatdata.log<-
log10(as.matrix(heatdata.meta.means.mat.short+1)) #Calculate the
log of the values. The +1 is for avoiding the error in the 0
values.

       #Select the list of pre-selected genes.
       ListGenes<- read.table("ListGenes.csv", header=TRUE,
sep=",") #Import the list of interesting known/guess genes

       #Object for filter the genes according to the category of
the genes.

keep.heatmap.Adhesion=ListGenes$Name[ListGenes$Catergory=="Adhesion
"]

       #Filter the data to select the different catergories.
       heatdata.log.filtered <-
heatdata.log[(row.names(heatdata.log)%in%keep.heatmap.Adhesion),]

       #Sort the data when we don't want to cluster
       heatdata.log.filtered <-
heatdata.log.filtered[order(row.names(heatdata.log.filtered)), ]
       heatdata.log.filtered <-
heatdata.log.filtered[,c("LB","GM","Wheat","Plutella_24h","Plutella
_36h","Galleria") ]

       #heatmap without clustering and not reordering of the genes.
       heatmap.2(heatdata.log.filtered,
            trace="none",margin=c(8,13), density.info="none",
            col=magma,
            breaks=seq(0.8,3.3,by=0.05),
            dendrogram="none",
            Rowv=NULL, Colv =NULL, #It doesn't reorganize the
columns and the rows
             main= "heatmap")
```

```
#Orthologue analysis
#Program: OrthoFinder 2.3.3.
   #batch download
   curl --remote-name --remote-time Strain_protein.faa.gz

   #Run OrthoFinder
   python2.7 orthofinder.py -f Data/-S diamond -n
Orthofinder_Output
```

## 11.5. References


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# Chapter 4

Phylogenetically closely related pseudomonads isolated from arthropods show differential insect killing abilities and genetic variations in insecticidal factors.

**This chapter is in preparation for publication in Environmental Microbiology Journal.**

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# 1. Abstract

*Pseudomonas* bacteria belonging to the *P. protegens* and *P. chlororaphis* species can promote plant growth, control soil borne pathogens and pest insects. They are able to invade the insect gut, transmigrate into the hemocoel causing a systemic infection and death due to several factors e.g. toxins, chitinases, antimicrobials or two-partner secretion (TPS) systems. Most insecticidal *Pseudomonas* described so far were isolated from roots or soil, so it is still widely unknown if these species can naturally occur in arthropods and what their relationship with these animals is. Therefore, we searched for *P. protegens* and *P. chlororaphis* in arthropods collected in an agricultural field and neighboring grassland. We could isolate *P. protegens* and *P. chlororaphis* from healthy insect of different orders and from myriapods. Insect isolates were compared to plant isolates for biocontrol-, insecticidal- and host colonization abilities. Our results from feeding assays with *Plutella xylostella* indicate that neither the origin of isolation nor the phylogenetic position are determining factors for the degree of insecticidal activity. *P. protegens* strains turned out to be homogeneous regarding biocontrol and insecticidal capabilities whereas *P. chlororaphis* strains were phylogenetically and phenotypically more heterogenous. A genotypic and phenotypic analysis of five very closely related *P. chlororaphis* isolates displaying varying levels of insecticidal activity revealed mutations in genes affecting the protein structure and function of several insecticidal factors i.e. the Fit toxin, a chitinase and TPSA probably leading to the reduction in insecticidal activity observed for some of the isolates. Our findings point towards an adaption to insects within

closely related *Pseudomonas* groups and make an important contribution to understand the ecology of insecticidal *Pseudomonas*.

## 2. Introduction

The *Pseudomonas* genome is complex and plastic, which allows these bacteria to colonize a wide variety of habitats such as plant-roots, animals or polluted water [1, 2]. Different *Pseudomonas* species are either generalists or form specific interactions with certain hosts. For instance, human pathogenic multidrug resistant *P. aeruginosa* strains are more adapted to cystic fibrosis patients than to healthy humans [3, 4], whereas different *P. syringae* pathovars are able to infect and cause disease in specific plant hosts [5, 6]. Some *Pseudomonas* are also adapted to live as endophytes which allows the bacteria to more efficiently exploit the resources of the plant host [7]. Also, *P. stutzeri* and *P. fluorescens* show preferences for colonizing roots of certain plant species over others [8].

*Pseudomonas* have been detected within the arthropod microflora in several studies [9, 10]. Insect microbiomes include bacteria, archea, fungi, viruses and protists which can protect the host against invading pathogens, increase the availability of nutrients [10–13] or even confer resistance to pesticides [14, 15]. Physiology, diet, behaviour and environment of the different insect orders highly influence the composition of the microbiota and its transmission [9, 10, 12, 16]. Here, we will focus on holometabolous insects like Coleoptera, Diptera and Lepidoptera. They mostly obtain their microbial communities from their diet or the environment and the communities change through the different life stages of the insect [17–25]. Yet, Diptera and Coleoptera retain some core microbiota that is transmitted vertically through generations [21, 26–28]. On the other hand, it has been proposed that Lepidoptera larvae do not have a core microbiota probably due to the demanding conditions of their guts such as high pH, antimicrobial peptides and the lack of pouches or cavities that can host microbial communities [12, 29]. Indeed, it has been shown that the Lepidoptera larvae obtain their microbiota from their diet and interactions with the soil [16, 29]. Other arthropods such as myriapods have not been studied as detailed as insects but one study described

*Proteobacteriaceae* associated to centipedes belonging to the Geophilidae family [30]. Although pseudomonads were detected in field and laboratory reared Lepidoptera [18, 31, 32] and Coleoptera [33, 23, 24], and laboratory reared Diptera [34], it is still uncertain whether they have established a relationship with these insects of if they are just transient bacteria in the gut.

Some plant beneficial fluorescent pseudomonads belonging to the *P. protegens*, *P. chlororaphis* and *P. fluorescens* species can also become pathogens of diverse pest insect species [35]. *P. protegens* and *P. chlororaphis* are known root colonizers and they can control soil-borne fungal pathogens [36]. However, they can, after oral uptake, also invade the insect gut, transmigrate into the hemocoel, cause a systemic infection and kill the insect [35, 37]. Insecticidal activity in these two species was firstly related to the Fit toxin encoded by the *fitD* gene [38]. This toxin is very similar to the Mcf (make caterpillars floppy) toxin from *Photorhabdus luminescens* and *Xenorhabdus nematophila* but is only partially responsible for the toxicity [39, 40]. Insecticidal activity, mostly studied in *P. protegens*, is a multifactorial trait that involves many different elements such as lipopolysaccharide O-antigens [41], the type VI secretion system [42], the cyclic lipopeptide orfamide A [43, 44], a chitinase and a phospholipase C [45], hydrogen cyanide [43], the toxins rhizoxin [46] or IPD072Aa [47] and two-partner secretion systems (thesis chapter 3, [48]).

*P. chlororaphis* and *P. protegens* cause high levels of mortality in larvae of different Lepidoptera species such as *Spodoptera littoralis*, *Pieris brassicae, Manduca sexta, Plutella xylostella* and *Heliothis virescens* [37–40, 49] and they also negatively affect pupation rates in *Delia radicum* [37]. In particular, *P. protegens* CHA0 is able to persist throughout different *D. radicum*, *P. xylostella* and *P. brassicae* life stages and can even be transmitted to new host plants by *D. radicum* flies [37]. However, no oral effect was found for larvae of the Coleoptera *Melolontha melolontha, Diabrotica balteata*, *Tenebrio molitor* and *Otiorhynchus sulcatus* even

though *P. protegens* CHA0 was detected in larvae, pupae and adults [37, 49].

Most of the published *P. protegens* and *P. chlororaphis* isolates with described insecticidal activity were isolated from soil or plant environments. Thus, the first aim of this study was to isolate insecticidal *P. protegens* and *P. chlororaphis* from arthropods collected in agricultural and neighbouring undisturbed grassland in order to verify their natural association with these animals. The second aim was to investigate whether root and arthropod isolates differ in their abilities or efficacies to colonize and kill an insect and to colonize plant roots and to protect them against a soilborne pathogen. Finally, the genetic background of the observed differences was assessed by a SNP calling. This study shows that *P. protegens* species are more homogeneous whereas *P. chlororaphis* are more phylogenetic and phenotypic diverse for the studied traits independently of their isolation origin.

# 3. Material and Methods

## 3.1. Sample collection

To assess the natural occurrence of insecticidal *P. protegens* and *P. chlororaphis*, arthropods, soil and plant-roots were collected in spring 2016 and spring 2017 in an agricultural field (wheat in 2016, potato in 2017) and a neighboring undisturbed grassland area. The field is located at Agroscope Reckenholz, Zurich, Switzerland Reckenholz (47º 25' 32'' N; 8º 30' 57'' E). Arthropod, root and soil samples were collected up to 50 cm of soil depth. The collected arthropods were visually classified as detailed as possible.

*Arthropod samples*: arthropods were surface disinfected first with 96% ethanol and then with 70% ethanol (20 s each). After each disinfection step, the animals were rinsed with distilled $H_2O$ amended with 0.05% SDS. The animals were homogenized in 1 ml or 15 ml 0.9% NaCl depending on their size with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland).

*Root samples*: the soil attached to the roots was removed with tap water and from 0.5 to 2 g of roots were incubated in 50 ml of 0.9% NaCl solution overnight at 3 ºC. Roots were shaken for 30 min at 3 ºC and 350 rpm to detach the bacteria from the root surface.

*Soil samples*: 20 g of sieved soil were suspended in 100 ml of 0.9% NaCl and shaken for 3 h at 150 rpm and 3 ºC.

## 3.2. Bacterial isolation and screening for insecticidal *Pseudomonas*

*Screening for Fit+ pseudomonads.* Undiluted and serially diluted arthropod-, root- and soil suspensions were plated on the *Pseudomonas* isolation medium King's B supplemented with ampicillin, 40 µl $ml^{-1}$; chloramphenicol, 13 µl $ml^{-1}$; and cycloheximide, 100 µl $ml^{-1}$ (KB+++) and kept at 24 ºC [50, 51]. Growing colonies were picked and regrown to separate single clones. A single colony from each isolate was picked and cultured overnight at 24 ºC in

lysogeny broth (LB, [52]), diluted and used for colony PCR with the DreamTaq polymerase (ThermoFisher Scientific, MA, USA). To detect insecticidal *Pseudomonas*, colony PCR was first performed on all isolated strains using a *Pseudomonas*-specific 16S rRNA primer (forward 5'-ACT TTA AGT TGG GAG GAA GGG-3'; reverse 5'-ACA CAG GAA ATT CCA CCA CCC-3'; annealing temperature 60 ºC; [53]) and positive strains were then screened using a *fitD*-specific primer (forward 5'- CTA TCG GGT SCA GTT CAT CA-3'; reverse 5'- TTC TTG TCG GSA AAC CAC T -3'; annealing temperature 60 ºC; [43]) All strains were stored in 43 % glycerol at -80 °C.

*Identification of P. chlororaphis and P. protegens.* DNA from *fitD*-positive *Pseudomonas* strains was extracted with Wizard Genomic DNA purification Kit (Promega, WI, USA) and a bigger fragment of the 16S rRNA was amplified using the following primers: forward 5'- AGA GTT TGA TCM TGG CTC AG -3'; reverse 5'- TAC GGY TAC CTT GTT ACG ACT T -3' and annealing temperature of 55 ºC [54]. The PCR product was purified with the FastAP Thermosensitive Alkaline phosphatase and the Exonuclease I (ThermoFisher Scientific, MA, USA). The sequencing reaction was set-up with the purified PCR product using the Big Dye v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and further cleaned with Sephadex GM-50 (GE-Healthcare, MA USA). PCR fragment sequencing was performed by Sanger sequencing following manufacturer's instructions (3130xl DNA analyzer, Applied Biosystems, CA, USA) to identify *P. chlororaphis* and *P. protegens*.

*Phylogenetic tree*. The phylogenetic tree was constructed on the concatenation of *gyrB*/*rpoD*/*rpoB* genes. Nucleotide sequences of these genes were extracted from the genomes (Supplementary Table S1). These nucleotide sequences were aligned using MUSCLE [55], concatenated (total of 8344 bp) and used to compute a Maximum Likelihood tree using PhyML [56]. The robustness of the tree was assessed with 100 bootstraps.

### 3.3. *P. protegens* and *P. chlororaphis* genome sequencing

The DNA from 10 *P. chlororaphis* and 13 *P. protegens* was extracted with Wizard Genomic DNA purification Kit (Promega, WI, USA) and the genome sequenced by MiSeq (2x300 paired-end v3 with Nextera XT library kit, Illumina, CA, USA) or DNA was extracted with the MagAttract high-molecular-weight (HMW) DNA kit (Qiagen, Germany) and sequenced by PacBio (SMRT v3, SMRTbell template preparation kit v1.0, Pacific Biosciences, CA, USA). The *de novo* genome assembly was performed using MIRA v4.0.2 with standard settings in accurate mode (MiSeq-sequenced strains) or RS_HGAP_Assembly.4 protocol in SMRT Link v6.0 for (PacBio sequenced strains). Genomes will be deposited in NCBI (submission reference SUB7300345).

GC content, average nucleotide identity and tetranucleotide analysis indexes were assessed with JSpecies v1.2.1 and are listed in Table S2, which also indicates DNA extraction method and sequencing technology for each strain.

### 3.4. Isolate characterization

*Distribution of insecticidal and plant-beneficial traits within the Pseudomonas fluorescens species complex*. A total of 126 amino acid or nucleotide sequences were screened by tBLASTn or BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A function was considered present inside a genome when showing at least 70% of identity over 70% of the length of the amino acid sequences. For very close orthologous functions (i.e. for orfamide, sessilin, viscocin, massetolide and other cyclic lipopeptides), the threshold for presence was set at least 80% of identity over 90% of the length of the nucleotide sequence.

*Insecticidal activity and colonization*

*Plutella xylostella* feeding assays and bacterial insect colonization. To assess the

insecticidal activities of the new isolates, a *P. xylostella* feeding assay was performed as described in detail in Flury et al., [37]. Briefly, *P. xylostella* eggs obtained from Syngenta Crop Protection AG (Stein, Switzerland) were reared at 25 ºC, 60% of humidity and 16 h/8 h day (162 µmol m-2s-1)/night cycle). Second instar larvae (about 5 days old) were fed with artificial diet pellets spiked with 10 ul of 0.9% NaCl or cell suspensions of the different bacterial strains ($OD_{600}$= 0.5 = 4 x $10^8$ cfu/pellet). Cell suspensions were prepared with washed cells from overnight Lysogeny broth cultures. Larvae were kept in 128-well bioassay trays (Frontier Agricultural Sciences, Delaware, USA) to avoid cannibalism at the same conditions used for rearing. Survival was regularly assessed by poking the larvae with a tip. Survival curves of different treatments were compared using the log-rank test ($p$-value<0.05) from the "survival 3.1.8" package (https://github.com/therneau/survival, [57] of R 3.6.0 (www.r-project.org). Statistical differences were calculated for the individual experiments but pooled experiments were plotted in a Kaplan-Meier graph as well to show an overview of all the experiments together. LT50 values were estimated using the "ecotox 1.4.1" R package (https://www.rdocumentation.org/packages/ecotox/versions/1.4.1) and considered significantly different when the 95% intervals were not overlapping between the strains. All LT50 values are included in Supplementary Table S3 and p-values of the log-rank test in Supplementary Table S4. All strains were tested at least twice in independent experiments. In total, 16 experiments were performed. In every experiment reference strains *P. chlororaphis* PCL1391 and *P. protegens* CHA0 were included as an internal standard.

A subset of larvae in the feeding assays was used to assess the strains ability to colonize insects. Five larvae per treatment were collected at different time points (5 h and 30 h for *P. protegens* strains and 5 h, 30 h and 45 h for *P. chlororaphis* strains) and washed twice in 70% EtOH for 20 s with subsequent rinsing with $H_2O$ amended with 0.05% SDS. Insects were homogenized in 1 ml of 0.9% NaCl with a Polytron PT-DA 2112 blender (Kinematica, Littau,

Switzerland). Homogenates were serially diluted and plated onto KB+++ medium. Colony forming units (CFU) were assessed after incubating plates for 2 days at 27 ºC. There were no statistical differences between the experiments (Kruskal-Wallis $p$-value<0.05), therefore, the data were pooled. Statistical differences in colonization numbers among strains were assessed using a Dunns's Test ($p$-value<0.05) from the "FSA" R package (https://github.com/droglenc/FSA). In the boxplot graphs, boxes correspond to the 25$^{th}$ and 75$^{th}$ percentiles, lines inside the boxes indicate the median and whiskers correspond to 1.5 times the interquartile range.

*Root colonization and biocontrol ability in natural soil.*

The ability of isolated strains to colonize roots and to protect plants against a root pathogen was tested in a cucumber pot assay with natural soil and the oomycete pathogen *Pythium ultimum.* Natural soil collected at the sampling site in Reckenholz (Agroscope, Switzerland) was used because we wanted to test the root colonization ability and disease suppressive capacity of the new isolates under competitive conditions i. e. in the presence of native soil microorganisms.

Cultivation of pathogen, bacteria and plants: *Pythium ultimum* strain Pu-11 was grown on a malt agar plate at 18 °C for 7 days. Three plugs with fungal mycelium were transferred to twice-autoclaved millet (organic millet, Migros, Switzerland) and the pathogen was cultivated at 18 °C for another seven days. *Pseudomonas* isolates were grown overnight in LB at 180 rpm and 24 °C. From these cultures, aliquots of 200 µl were spread onto KB+++ plates and incubated at 27 ºC for 24 h. Bacteria were scraped from plates and washed with 0.9% NaCl. Suspensions used for pot inoculation were adjusted to an $OD_{600}$ of 4.0 (approx. 3.2 x $10^9$ cells/ml). Cucumber seeds of the variety "Chinese Snake" (Bigler Samen AG, Thun, Switzerland) were sterilized in 1.4% NaClO for 30 min, washed thoroughly with sterile water

and left to germinate on wetted filter paper for 1.5 days at 24 ºC in the dark.

Set up of pot experiments: 250 ml pots were filled with 320 g of a 4:1 soil/quartz sand (1.5 – 2.2 mm diameter) mixture. Each pot received 1 g of the *P. ultimum*-millet inoculum. Control treatments without pathogen received 1 g of autoclaved *P. ultimum*-millet mixture. The pathogen inoculum was thoroughly mixed into the soil. Then, 5 ml of a *Pseudomonas* suspension were added to pots resulting in a final concentration of $10^7$ cells/gram of soil. Control treatments were amended with the same volume of 0.9% NaCl. Finally, pots received 45 ml of sterile water and three cucumber seedlings were planted per pot. Plants were grown for 10 days in a growth chamber with a 16h/8h day (210 mmol $m^2$ $sec^{-1}$)/night period at 22 ºC/20 ºC and 70% of humidity. Six replicates with and six without pathogen were prepared per bacterial strain. Two independent experiments were performed over time.

Evaluation of pot experiments: Nine days after inoculation, plant mortality and disease severity were assessed (see Supplementary Figure 1 for disease classification) and shoots were weighted. Roots were washed, weighted and incubated overnight in 50 ml of autoclaved NaCl solution (1 %) at 3 ºC. The next day, roots were shaken at 400 rpm and 3 ºC for 30 min to detach the bacteria from the root surface. Serial dilutions of root suspension were plated onto KB+++ and CFU numbers were assessed after two days of incubation at 27 ºC.

Since the factor experiment had a significant impact on root colonization and shoot weight data (Kruskal-Wallis $p$-value<0.05), the data of individual experiment could not be pooled. Data were first subjected to Shapiro-Wilk normality test ($p$-value<0.05) to check normal distribution. Differences in root colonization were assessed with one-way ANOVA ($p$-value<0.05) for experiments with normally distributed data (both *P. protegens* exps. and the second *P. chlororaphis* exp.) or Kruskal-Wallis ($p$-value<0.05) if data were not normally distributed (first *P. chlororaphis* exp.). Shoot weight data of all experiments were not normally distributed, therefore, a Dunn's Test ($p$-value<0.05) was used to assess the differences between

the shoot weights of plants treated with the different *Pseudomonas* strains. Kruskal-Wallis and ANOVA test are part of the "stats" package built-in within R. In the boxplot graphs, boxes correspond to the 25$^{th}$ and 75$^{th}$ percentiles, lines inside the boxes indicate the median and whiskers correspond to 1.5 times the interquartile range.

### 3.5. SNP calling

To estimate the genetic differences between the characterized strains and their reference (*P. chlororaphis subsp. piscium* PCL1391, accession number NZ_CP027736.1), Snippy was used with the following command indicating that the input were contigs and not raw reads: snippy --report --outdir Output/Sample --ref Reference_Strain.gbk --ctgs Sample.fasta.

To check the homogeneous distribution of the mutations across the genome, each affected locus was plotted in R. Afterwards, a principal component analysis (PCA) was performed using Tassel 5 [58] with the total amount of mutations to observe the differences between the *P. chlororaphis* PCLAR01, PCLAR04 and PCLAR03 strains. Non-synonymous mutations were selected to observe which genes had the highest content of changes among the strains. Specific regions corresponding to the genes C4K33_RS18300 (*fitD*), C4K33_RS15780 (*plcN*), C4K33_RS10170 (*chiD*), C4K33_RSA19905 (*tpsA2*), C4K33_RS08790 (*tpsA1,3*) and C4K33_RS21280 (*tpsA4*) were analyzed more in detail. To check if any of the mutations were affecting the predicted protein domains, the nucleotide sequence of the genes was used to find conserved domains in the Conserved Domain database of the NCBI (www.ncbi.nlm.nih.gov/cdd/).

# 4. Results and Discussion

## 4.1. *P. protegens* and *P. chlororaphis* strains are naturally associated with arthropods, plants and soil of agricultural fields

*P. protegens* and *P. chlororaphi*s have demonstrated insecticidal activity [37–39, 43, 45, 49, 59–61]. However, it is not known, whether these two species are naturally associated with insects. The first aim of our study was thus to search for presence of *P. protegens* and *P. chlororaphis* in insects and other arthropods collected from soil. We collected 120 arthropods of 51 different genera in two years at the same site i.e. an agricultural field (wheat in 2016, potato in 2017) and a patch of neighbouring grassland. After surface disinfection, a total of 94 pseudomonads were isolated from those arthropods. Fourteen were identified as *P. protegens* and 16 as *P. chlororaphi*s based on their 16s RNA sequences clustering with the respective phylogenetic subgroups by a multilocus sequence analysis (data not shown). Additionally, 7 *P. chlororaphis* were isolated from grassland or potato roots or soil. Ten isolates of each species were sequenced and included in the study presented here. The GC content and TETRA and ANIm indexes compared to *P. chlororaphis* PCL1391 and *P. protegens* CHA0 are shown in Supplementary Table S2. Interestingly all *P. protegens* strains derived from these two samplings in the two years were isolated from arthropods and we did not detect this species in the root or soil samples we collected at the same site (Table 1). The *P. protegens* isolates are closely related to each other but diverge from other previously described root isolates (Fig. 1). The isolates cluster together depending on the isolation year with one exception of a 2017 isolate, which clusters together with the 2016 isolates. The 2017 isolates might be the descendant generations of the strains found in 2016 that adapted to the conditions of a new crop. In contrast to *P. protegens*, *P. chlororaphis* could be isolated from roots, soil and invertebrates. In the phylogenetic tree based on the *gyrB*/*rpoD*/*rpoB* complete genes, the new *P. chlororaphis* isolates cluster together either with *P. chlororaphis* ssp. *piscium* type strain

DSM 21509$^T$, *P. chloraphis* subsp. *aureofaciens* type strain LMG1245, or *P. chlororaphis* subsp. *piscium* strain PCL1391 (Fig. 1). *P. chlororaphis* isolates clustered independently of the year and niche (soil, root, arthropod) of isolation (Table 1, Fig. 1).

**Table 1: *P. chlororaphis* and *P. protegens* strains included in the phenotypic characterization of this study.**

| Species | Strain | Isolation | Country | Year of isolation | Reference |
|---|---|---|---|---|---|
| *P. chlororaphis* | PCL1391 | Tomato root | Spain | | [62] |
| *P. chlororaphis* | 30.84 | Wheat seed | | | [63] |
| *P. chlororaphis* | PCLAR01 | *Aphodiinae* (Coleoptera) | Agroscope, Switzerland | 2016 | This study |
| *P. chlororaphis* | PCLAR02 | *Aphodiinae* (Coleoptera) | Agroscope, Switzerland | 2016 | This study |
| *P. chlororaphis* | PCLAR03 | Diptera | Agroscope, Switzerland | 2017 | This study |
| *P. chlororaphis* | PCLAR04 | *Scarabaeidae* pupa (Coleoptera) | Agroscope, Switzerland | 2017 | This study |
| *P. chlororaphis* | PCLAR05 | *Scarabaeidae* larva (Coleoptera) | Agroscope, Switzerland | 2017 | This study |
| *P. chlororaphis* | PCLRT01 | Grass land, root | Agroscope, Switzerland | 2016 | This study |
| *P. chlororaphis* | PCLRT02 | Potato root | Agroscope, Switzerland | 2017 | This study |
| *P. chlororaphis* | PCLRT03 | Potato root | Agroscope, Switzerland | 2017 | This study |
| *P. chlororaphis* | PCLRT04 | Potato root | Agroscope, Switzerland | 2017 | This study |
| *P. chlororaphis* | PCLSL01 | Potato field, soil | Agroscope, Switzerland | 2017 | This study |
| | | | | | |
| *P. protegens* | CHA0 | Tobacco root | Switzerland | | [64] |
| *P. protegens* | K94.41 | Cucumber root | | | [65] |
| *P. protegens* | Pf-1 | Tobacco root | Morens, Switzerland | | [66] |
| *P. protegens* | Pf-5 | Soil | USA | | [61] |
| *P. protegens* | PGNL1 | Tobacco root | Ghana | | [66] |
| *P. protegens* | PGNR2 | Tobacco root | Ghana | | [66] |
| *P. protegens* | PPRAR01 | *Lithobius* (Myriapod) | Agroscope, Switzerland | 2016 | This study |
| *P. protegens* | PPRAR02 | *Lithobius* (Myriapod) | Agroscope, Switzerland | 2016 | This study |
| *P. protegens* | PPRAR03 | Lepidoptera larvae | Agroscope, Switzerland | 2016 | This study |
| *P. protegens* | PPRAR04 | Agriotes (Coleoptera) | Agroscope, Switzerland | 2016 | This study |
| *P. protegens* | PPRAR05 | *Staphylinidae* (Coleoptera) | Agroscope, Switzerland | 2016 | This study |
| *P. protegens* | PPRAR06 | Agriotes (Coleoptera) | Agroscope, Switzerland | 2017 | This study |
| *P. protegens* | PPRAR07 | *Lithobius* (Myriapod) | Agroscope, Switzerland | 2017 | This study |
| *P. protegens* | PPRAR08 | *Geophilidae* (Myriapod) | Agroscope, Switzerland | 2017 | This study |
| *P. protegens* | PPRAR09 | *Curculionidae* (Coleoptera) | Agroscope, Switzerland | 2017 | This study |
| *P. protegens* | PPRAR10 | *Agrypnus murinus* (Coleoptera) | Agroscope, Switzerland | 2017 | This study |

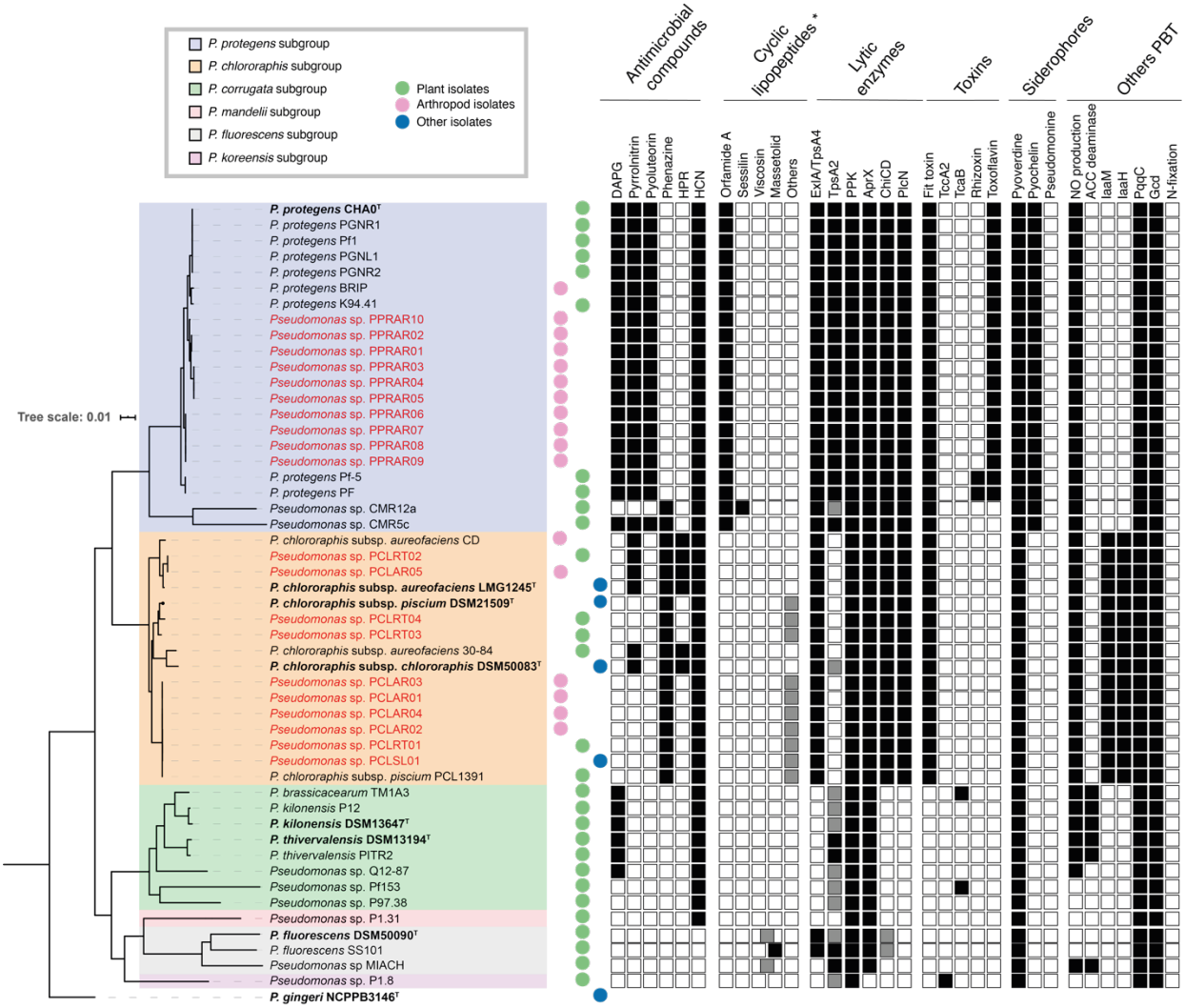


**Figure 1: Distribution of functional traits related to insecticidal and/or plant-beneficial activity among fluorescent *Pseudomonas*.** The phylogenetic tree of 54 *Pseudomonas* genomes is based on the concatenation of *gyrB*/*rpoD*/*rpoB* genes. The squares correspond to the presence of amino acids or DNA sequences related to a functional trait. Black squares: present; grey squares: potentially present; white squares: absent. Type strains are marked in bold. The isolates described in this study are marked in red. The isolation site is indicated as follows: pink for arthropod isolates; green for plant isolates; blue for other isolates.

*Pseudomonas* have been associated with numerous arthropods classes, especially insects [9, 10]. Insecticidal *Pseudomonas* were isolated from laboratory reared *Schistocerca gregaria* Forskål (Orthoptera) [32] and field collected *Spodoptera litoralis* larvae (Lepidoptera) [31]. Non-insecticidal *Pseudomonas* were also found in *Oberea linearis* larvae (Coleoptera) collected in the field [33]. However, none of these studies characterized the detected pseudomonads at the species level. Thus, information about *P. protegens* and *P. chlororaphis* associated to arthropods is scarce. The only so far characterized *P. protegens* and *P.*

*chlororaphis* originating from arthropods were strains BRIP and CD, respectively, isolated from cyclops (Maxillopoda) [45, 59]). This study is therefore, to our best knowledge, the first describing and characterizing arthropod-isolated bacteria belonging to these two species.

The new *P. protegens* strains isolated in this study, all arthropod isolates, harbour the same traits as other *P. protegens* strains in our collection and further isolates published by other groups, which were mainly isolated from soil or plants. This confirms that the distribution pattern of specific insecticidal and plant-beneficial traits is very consistent within this *Pseudomonas* subgroup (Fig. 1). An exception is rhizoxin, a toxin with insecticidal activity so far only detected in a specific phylogenetic clade within the *P. protegens* subgroup harbouring strains Pf-5 and PF (Fig. 1). This toxin is absent in the new isolates which is in line with their phylogenetic position. The *P. chlororaphis* subgroup is much more heterogeneous regarding distribution of the plant-beneficial/insecticidal traits shown in Fig. 1, but the new isolates possess the same traits as the other strains they are clustering with.

Our results indicate that plant beneficial *P. protegens and P. chlororaphis* are indeed commonly associated with healthy arthropods and that they harbour the same traits as closely related strains isolated from soil or roots.

### 4.2. *P. protegens* and *P. chlororaphis* isolates show variable insecticidal activity independent from their root or arthropod origin.

Insecticidal activity in *Pseudomonas* is a multifactorial trait [35, 37–39, 41, 43–46, 48, 61]. *P. chlororaphis/protegens* arthropod and root isolates seem to harbour the same pattern of insecticidal traits (Fig. 1). Still, we wondered whether they would differ in insecticidal activity. To analyse the insecticidal capabilities of our new strains, second instar *P. xylostella* larvae were fed with artificial diet pellets spiked with 4 x $10^6$ bacterial cells. Mortality was regularly assessed which allowed to calculate the LT50 and the survival rates of the larvae. LT50 and

mortality curves were compared to the well-described reference strains *P. protegens* CHA0 and *P. chlororaphis* PCL1391, both isolated from plant roots. These comparisons are summarised in Table 2 and fully depicted in Supplementary Table S3 and S4.

The performance of *P. protegens* strains was quite consistent as killing speed only differed occasionally compared to the reference *P. protegens* CHA0 (Table 2). On the other hand, *P. chlororaphis* strains displayed more variability as several of them killed significantly slower or faster compared to the reference strain PCL1391 (Table 2). This is clearly reflected in the Kaplan-Meier survival plots performed on all strains and experiments showing that larvae fed with *P. protegens* all died and at a similar pace (Supplementary Fig. S2A) whereas some *P. chlororaphis* strains kill much slower than others with particular isolates only causing partial mortality (Supplementary Figure S2B). Kaplan Meyer plots of individual experiments are shown in Supplementary Fig. S3 and according log rank values in Supplementary Table S4. The LT50 values presented in Supplementary Table S3 show a similar scenario with *P. protegens* strains differing much less among each other in the individual experiments than the *P. chlororaphis* strains. In general, *P. protegens* kills faster than *P. chlororaphis*, which was already observed by Flury et al., [45]. There were differences in larval susceptibility between the first sets of experiments (exps. 1 to 5) and the experiments performed one year later (exps. 6 to 16). In the first set, larvae died generally at a slower pace compared to the second, which was probably due to different fitness of larvae at different rearing times. LT50 values for larvae in the first set of experiments ranged from 37 to 70 h in the *P. protegens* and from 66 to 191 h in the *P. chlororaphis* treatments and in the second set from 20 and 37 h for *P. protegens* and from 20 to 57 h for *P. chlororaphis* (Supplementary Table S3).

**Table 2: Summary of comparisons of insecticidal activity of different *P. chlororaphis* and *P. protegens* isolates with the model reference strains *P. chlororaphis* PCL1391 and *P. protegens* CHA0.** Numbers indicate the number of experiments in which a strain was significantly more insecticidal (> or +), less insecticidal (< or -) or not significantly different (=) compared to PCL1391 (reference for *P. chlororaphis*) or CHA0 (reference for *P. protegens*). Comparisons are based on LT50 and log-rank tests performed on Kaplan Meyer survival curves. *P. xylostella* larvae were fed with pellets spiked with 4 x $10^6$ bacterial cells and survival was recorded over time. LT50 values were calculated using the "ecotox" package of R and considered significantly different if the 95% confidence intervals of the studied strains and references did not overlap. Insect survival curves were evaluated using the log rank test in the "survival" package of R and considered significantly different at $p < 0.05$. Detailed results of individual experiments are shown in Supplementary Figures S2 and S3 (Kaplan Meyer survival curves), Supplementary Table S3 (LT50 values) and Supplementary Table S4 ($p$ values of log-rank tests).

| | | | LT50 | | | Log-Rank Test | | | | Total Experiments |
|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Strain** | **Isolation** | < | = | > | + | = | - | = NaCl | |
| *P. chlororaphis* | PCL1391 | Tomato root | | | | | | | | Reference |
| *P. chlororaphis* | 30.84 | Wheat seed | | 1 | 1 | | | 2 | | 2 |
| *P. chlororaphis* | PCLAR01 | *Aphodiinae* (Coleoptera) | | | 3 | | | 3 | | 3 |
| *P. chlororaphis* | PCLAR02 | *Aphodiinae* (Coleoptera) | | | 2 | | | 2 | | 2 |
| *P. chlororaphis* | PCLAR03 | Diptera | 2 | 3 | | 1 | 4 | | | 5 |
| *P. chlororaphis* | PCLAR04 | *Scarabaeidae* pupa (Coleoptera) | | 1 | 5 | | | 3 | 3 | 6 |
| *P. chlororaphis* | PCLAR05 | *Scarabaeidae* larva (Coleoptera) | 1 | 2 | 1 | 1 | 2 | 1 | | 4 |
| *P. chlororaphis* | PCLRT01 | grass land, roots | | 2 | | 1 | 1 | | | 2 |
| *P. chlororaphis* | PCLRT02 | potato root | 3 | | | | 1 | 2 | | 3 |
| *P. chlororaphis* | PCLRT03 | potato root | | 1 | 2 | | 1 | 2 | | 3 |
| *P. chlororaphis* | PCLRT04 | potato root | 1 | | 2 | | 1 | 2 | | 3 |
| *P. chlororaphis* | PCLSL01 | potato field, soil | 1 | 1 | | | 1 | 1 | | 2 |
| | | | | | | | | | | |
| *P. protegens* | CHA0 | Tobacco root | | | | | | | | Reference |
| *P. protegens* | K94.41 | Cucumber root | 1 | 2 | | 1 | 2 | | | 3 |
| *P. protegens* | Pf-1 | Tobacco root | | 2 | 1 | | 2 | 1 | | 3 |
| *P. protegens* | Pf-5 | Soil | | 2 | | | 2 | | | 2 |
| *P. protegens* | PGNL1 | Tobacco root | | 2 | | | 2 | | | 2 |
| *P. protegens* | PGNR2 | Tobacco root | | 2 | | | 2 | | | 2 |
| *P. protegens* | PPRAR01 | *Lithobius* (Myriapod) | | 2 | 1 | | 2 | 1 | | 3 |
| *P. protegens* | PPRAR02 | *Lithobius* (Myriapod) | | 2 | 1 | | 2 | 1 | | 3 |
| *P. protegens* | PPRAR03 | Lepidoptera larvae | | 3 | | | 3 | | | 3 |
| *P. protegens* | PPRAR04 | Agriotes (Coleoptera) | | 3 | | | 3 | | | 3 |
| *P. protegens* | PPRAR05 | *Staphylinidae* (Coleoptera) | | 2 | 1 | 1 | 1 | 1 | | 3 |
| *P. protegens* | PPRAR06 | Agriotes (Coleoptera) | | 2 | | 1 | 1 | | | 2 |
| *P. protegens* | PPRAR07 | *Lithobius* (Myriapod) | | 2 | | 1 | 1 | | | 2 |
| *P. protegens* | PPRAR08 | *Geophilidae* (Myriapod) | | 1 | 1 | | 2 | | | 2 |
| *P. protegens* | PPRAR09 | *Curculionidae* (Coleoptera) | 1 | 1 | | 2 | | | | 2 |
| *P. protegens* | PPRAR10 | *Agrypnus murinus* (Coleoptera) | | 2 | | | 2 | | | 2 |

In summary, for both species root and arthropod isolates show similar levels of insecticidal activities. The variation observed for *P. chlororaphis* strains does not seem to be related to the phylogenetic position or the niche of isolation (plant, soil vs arthropod). Root isolates from crops or grassland showed similar variability in activity against *P. xylostella* larvae as did arthropod isolates of the *P. chlororaphis* species (Table 2).

To our knowledge, this is the first study comparing insecticidal activity of *P. protegens* and *P. chlororaphis* strains isolated from field-collected arthropods, with strains isolated from roots and soil. Our new strains were isolated from healthy myriapods or healthy insects belonging to different orders, mostly to Coleoptera. However, all of them were insecticidal in our feeding system with *P. xylostella,* even if they showed different progression of the infection. This might indicate species-specific adaptation; pseudomonads might have commensal interactions with certain arthropods and pathogenic relationships with others. This is supported by several studies describing different effects of *P. protegens* and *P. chlororaphis* on insects of different orders such as differences in mortality, in persistence or in causing anomalous morphologies in adults [35, 37, 60, 67]. In previous experiments, *O. sulcatus* [37], *D. balteata*, *T. molitor*, *M. melolontha* [49] and *Agriotes obscurus* (our unpublished data) larvae showed none or very little susceptibility upon feeding on diet or plants treated with *P. protegens* CHA0. Yet, the bacteria were able to persist through different life-stages in *O. sulcatus*, *M. melolontha* [37, 49] and *A. obscurus* (our unpublished data). In contrast, *P. protegens* CHA0 showed a slight toxicity to *O. sulcatus* adults in CHA0 feeding assays (Esther Fisher personal communication) and to *M. melolontha* and *T. molitor* when injected into the hemocoel [49]. Thus, we speculate that the association of *P. chlororaphis* and *P. protegens* strains with Coleoptera might be rather commensal and it only becomes pathogenic when the bacteria have access to the hemolymph. This might occur in already weakened animals that are injured or attacked by other diseases or predators. However, our experiments were conducted

with laboratory-reared Lepidoptera meanwhile the Coleoptera *O. sulcatus*, *M. melolontha* and *A. obscurus* were collected from the field. Under laboratory rearing conditions, the insects are not exposed to the nutrients and microorganisms of their natural habitat, which are important for the insect fitness and protection against invader microbes respectively [13, 68]. The intake of different diets affects the immune response of several insect species e.g. *Grammia incorrupta* and *Bombus terrestris* [68–70]. Thus, further quantitative studies with field-collected Lepidoptera and Coleoptera would be necessary to confirm pathogenic or commensal species- or order-specific interactions of *P. protegens* and *P. chlororaphis* with insects.

### 4.3. Closely related *P. chlororaphis* strains show greater phenotypic variability than *P. protegens* strains.

We wondered if the differences in mortality could be related to the ability to colonize the host. Therefore, we aimed at further characterizing a subset of strains: four *P. protegens* strains i.e. two root and two insect isolates and five *P. chlororaphis* strains i.e. two root isolates and three very closely related insect isolates (Fig. 1). Strains of the same species were compared to each other for insecticidal activity, insect colonization ability, root colonization ability and efficacy in controlling root disease caused by the oomycete pathogen *P. ultimum*.

To perform the insect assays, *P. xylostella* second instar larvae were fed with artificial diet pellets spiked with 4 x $10^6$ bacterial cells, mortality was monitored over time and, in parallel a subset of insects were extracted after 5 h, 30 h or 45 h. *P. protegens* isolates from root and from insects killed and colonized the larvae equally well in three experiments with only strain K94.41 displaying in one out of three experiments a faster killing speed compared to the reference strain CHA0 (Fig. 2 A, B, C, Fig. 3A). All strains were able to kill 100% of the larvae within 50 h. The mean insect colonization increased by 2 to 4 orders of magnitude between the two time points (5 h and 30 h) and reached around log 5 CFU per larvae after 30

h (Fig. 3A).

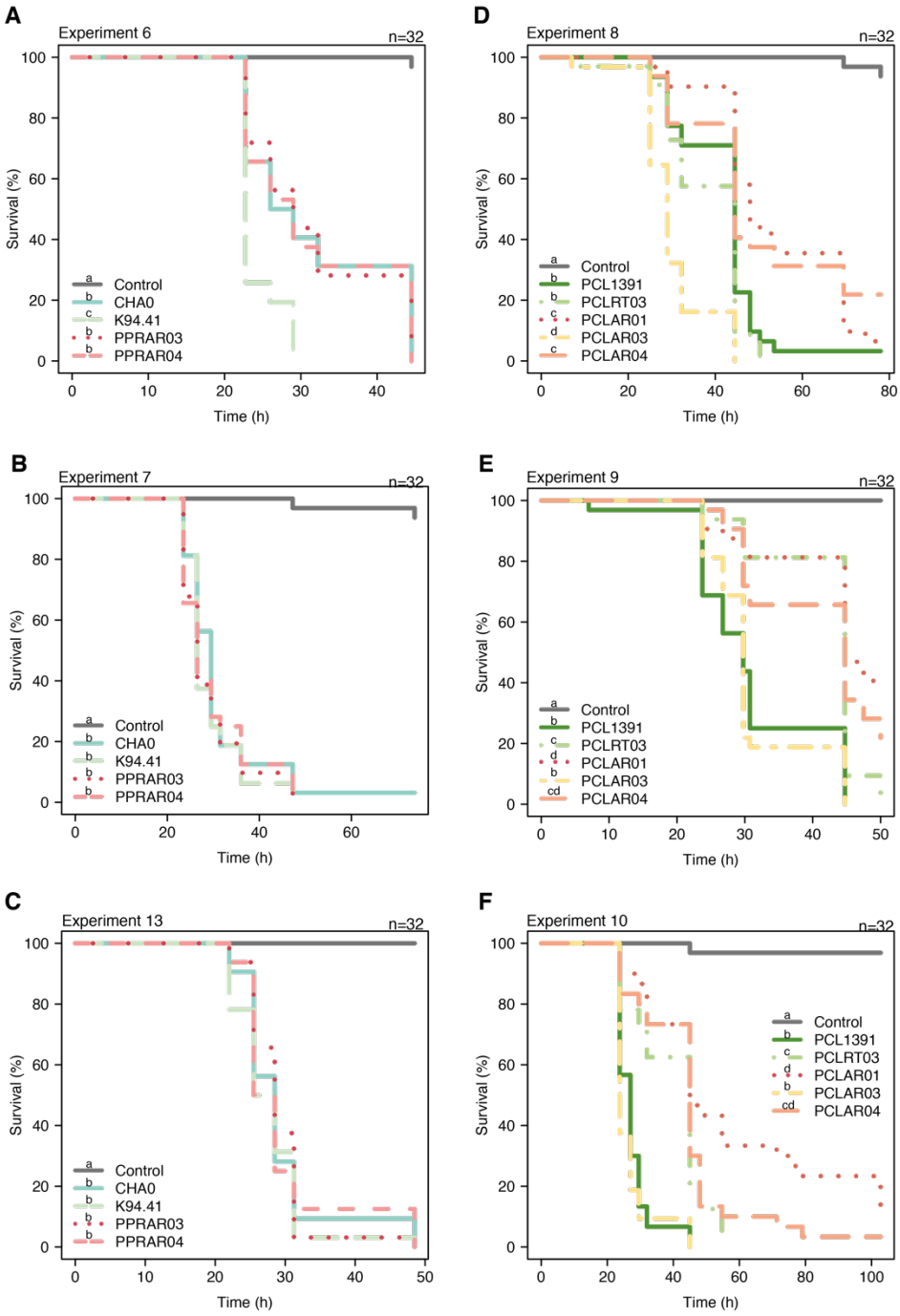


**Figure 2: Survival of *Plutella xylostella* larvae after oral uptake of different *Pseudomonas protegens* (A, B, C) and *Pseudomonas chlororaphis* (D, E, F) strains.** Second instar larvae of *P. xylostella* were fed with artificial diet pellets spiked with 4 x $10^6$ bacterial cells and mortality was assessed periodically by poking the insects. Strains were isolated from roots (*P. protegens* CHA0, *P. protegens* K94.41, *P. chlororaphis* PCL1391 and *P. chlororaphis* PCLRT03) or from arthropods (*P. protegens* PPRAR03, *P. protegens* PPRAR04, *P. chlororaphis* PCLAR01, *P. chlororaphis* PCLAR03 and *P. chlororaphis* PCLAR04). Statistical differences between the survival of the insects exposed to the different bacteria are depicted as different letters in the legend (Log-rank test $p < 0.05$). Experiment number and number of treated larvae is depicted on top of the figures.

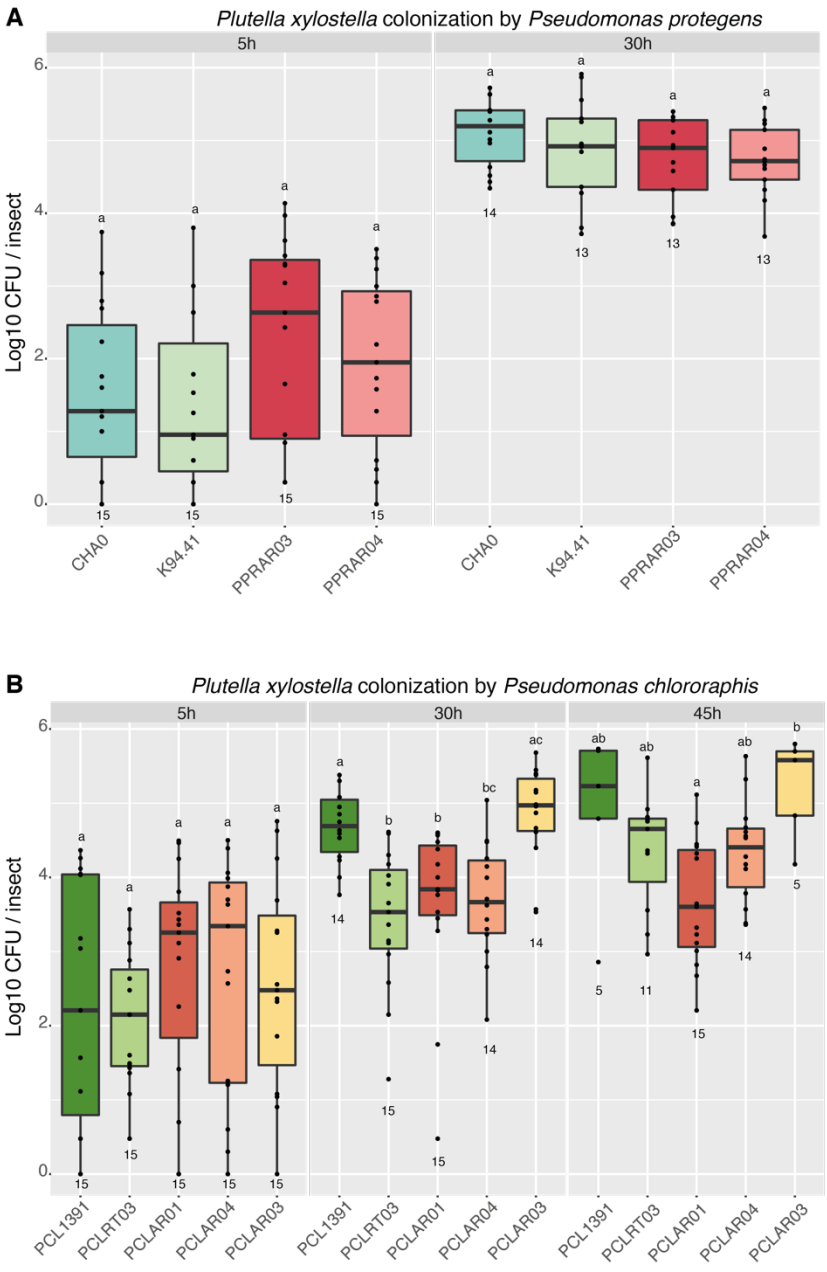


**Figure 3: Colonization of *Plutella xylostella* by *Pseudomonas protegens* (A) and *Pseudomonas clororaphis* (B) after oral uptake.** Second instar larvae of *P. xylostella* were fed with pellets of artificial diet spiked with 4 x $10^6$ bacterial cells. Larvae were surface sterilized and homogenated after 5, 30 or 45 h and serial dilutions of homogenates plated on selective medium. Strains were isolated from roots (*P. protegens* CHA0, *P. protegens* K94.41, *P. chlororaphis* PCL1391 and *P. chlororaphis* PCLRT03) or from arthropods (*P. protegens* PPRAR03, *P. protegens* PPRAR04, *P. chlororaphis* PCLAR01, *P. chlororaphis* PCLAR03 and *P. chlororaphis* PCLAR04). For each time point, significant differences between strains are indicated with different letters on top of the boxplots (Dunn's test $p < 0.05$). Numbers at the bottom of boxplots indicate numbers of extracted larvae.

In contrast, *P. chlororaphis* strains were more variable as the Coleoptera isolates (PCLAR01 and PCLAR04), closely related to PCL1391 in the phylogeny, displayed delayed killing speed in all three experiments. The potato root isolate (PCLRT03) killed slower twice and the Diptera isolate (PCLAR03) which is also very closely related to PCL1391 and the Coleoptera isolates, killed once significantly faster than PCL1391 (Fig. 2). The insect colonization dynamics shown in Fig. 3B nicely support the mortality curves as larvae infected with potato root and beetle isolates harboured lower bacterial cell numbers compared to the Diptera isolate and PCL1391.

For the plant assays cucumber seedlings were inoculated with different *Pseudomonas* strains and infected or not with the plant-pathogen *P. ultimum.* After 9 days, plants were assessed for disease severity, and fresh weight and root colonization were determined. None of the investigated strains had an impact on shoot weights in absence of the pathogen (Figures 4, 5). All *P. protegens* strains were capable to reduce disease in both experiments with a reduction of mortality ranging from 50% to 75% (Fig. 4AB) resulting in a significantly boosted shoot weight in both (strain PPRAR03) or one (all other strains) of the experiments (Fig. 4CD). Interestingly an insect isolate, PPRAR03 provided best plant protection against *P. ultimum* among the *P. protegens* strains. Root colonization slightly increased in presence of the pathogen but it was only significant for *P. protegens* strains CHA0 and PPRAR04 in one out of two experiments. When considering the same experiment and *P. ultimum* concentration, there were no significant differences between strains regarding root colonization (Supplementary Fig. S4). All *P. chlororaphis* strains reduced plant mortality (Fig. 5AB), especially in experiment two, however, this only resulted in significantly increased shoot weights for plants treated with strain PCLRT03 in the second experiment (Figs. 5AC). The stimulation of population sizes in presence of the pathogen was less consistent than observed for *P. protegens* but was significant for strains PCL1391 and PCLAR03 in one out of two experiments. There were no significant differences in root colonization between *P. chlororaphis* strains in absence of the pathogen. However, on *P. ultimum* infected roots, PCLAR01 established significantly higher population sizes in experiment two compared to PCLRT03 and PCLAR03 (Supplementary Fig. S5).

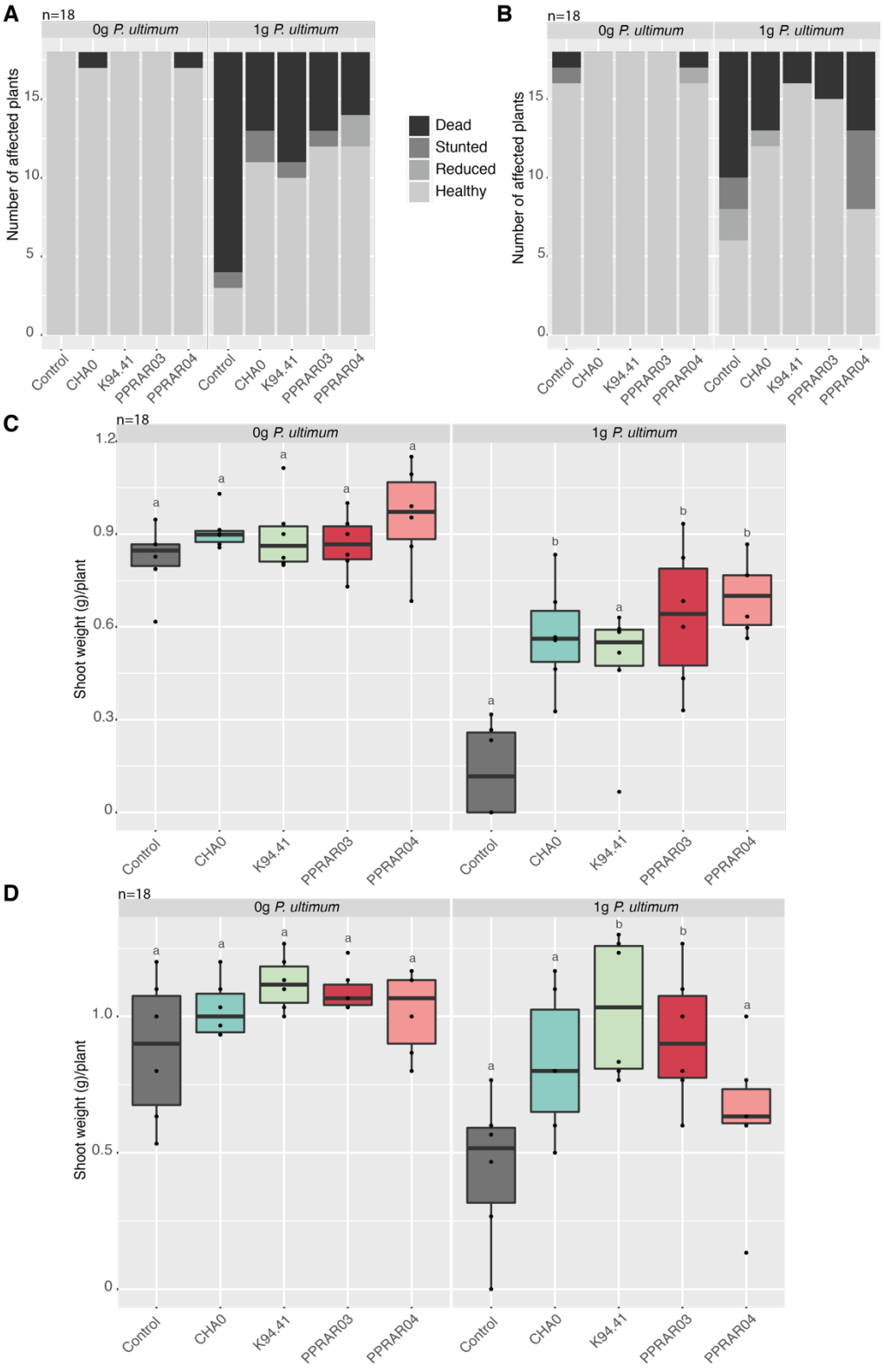


**Figure 4: Protection of cucumber plants against *Pythium ultimum* by *Pseudomonas protegens* root and insect isolates**. Phenotype (A, B) and shoot weights (C, D) of 10-day-old cucumber plants inoculated with different *P. protegens* strains and infected (1 g) or not (0 g) with *P. ultimum*. A, C = experiment one; B, D = experiment two. Strains were isolated from roots (*P. protegens* CHA0 and *P. protegens* K94.41) or from insects (*P. protegens* PPRAR03 and *P. protegens* PPRAR04). Significant differences between strains are indicated with different letters on top of the boxplots (Dunn's test, $p < 0.05$). A and C: experiment one; B and D: experiment two.

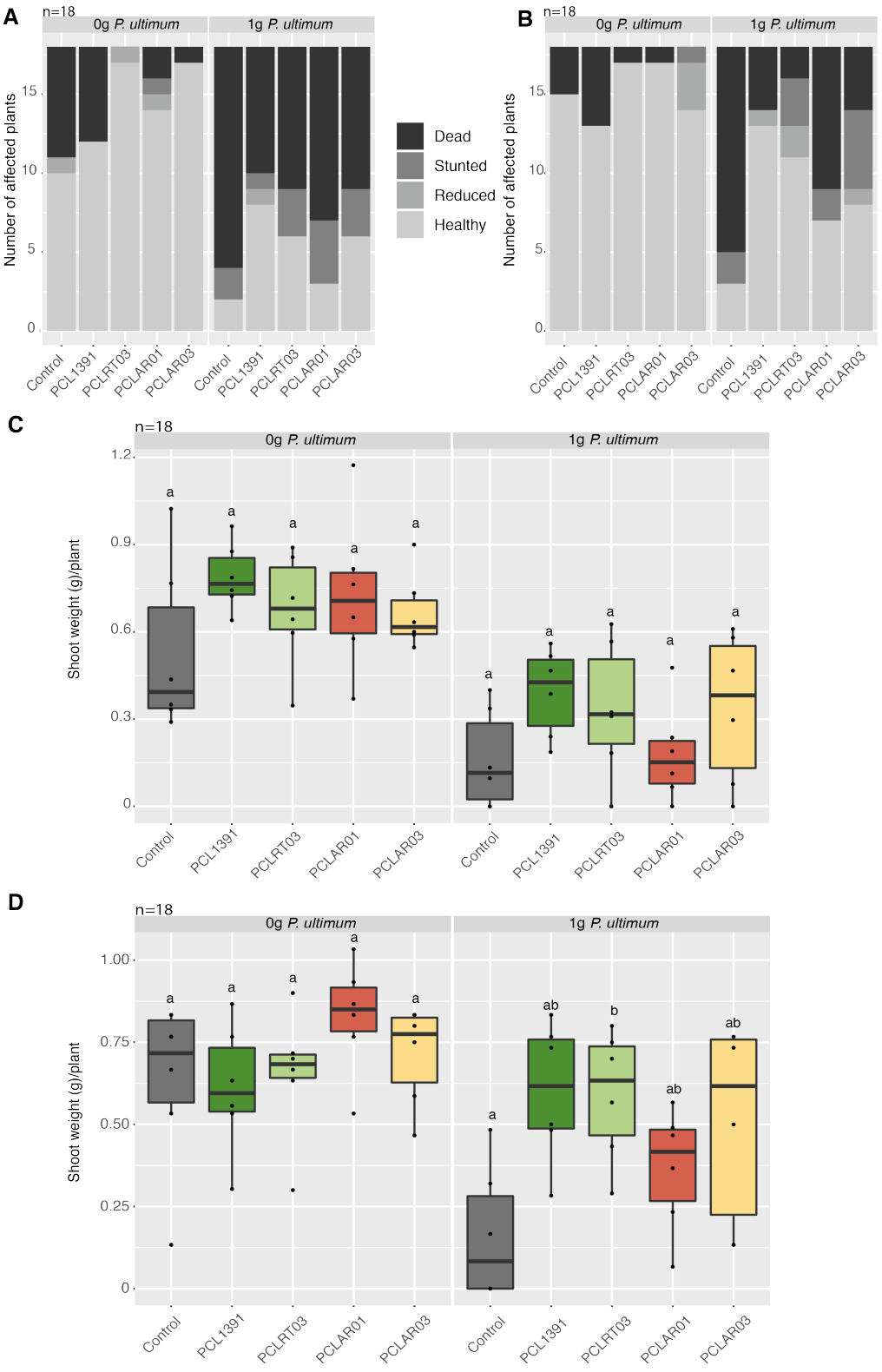


**Figure 5: Protection of cucumber plants against *Pythium ultimum* by *Pseudomonas chlororaphis* root and insect isolates**. Phenotype (A, B) and shoot weights (C, D) of 10-day-old cucumber plants inoculated with different *P. chlororaphis* strains and infected (1 g) or not (0 g) with *P. ultimum*. A, C = experiment one; B, D = experiment two. Strains were isolated from roots (*P. chlororaphis* PCL1391 and *P. chlororaphis* PCLRT03) or from insects (*P. chlororaphis* PCLAR01 and *P. chlororaphis* PCLAR03). Significant differences between strains are indicated with different letters on top of the boxplots (Dunn's test $p$-value<0.05). A and C: experiment one; B and D: experiment two.

In summary, the studied *P. protegens* isolates performed equally well on plants and in insects. In contrast, *P. chlororaphis* strains were quite homogenous in colonizing roots and suppression of the root pathogen but showed much more variability regarding insect colonization and oral activity. Our findings showed that, among three phylogenetically very closely related insect isolates, the two beetle isolates (PCLAR01 and PCLAR04) were less efficient *P. xylostella* colonizers/killers than the Diptera isolate. This might point towards a certain adaptation to different insect species/orders as discussed in the previous chapter.

Plant growth promotion and biocontrol of soil-borne fungal pathogens such as *P. ultimum*, have been widely described in *P. protegens* and *P. chlororaphis* and in recent years their insect associated life style was discovered and explored [35–37, 40, 44–46, 48, 61, 71, 72]. The results presented here reveal that members of the species *P. protegens* and *P. chlororaphis* disregarding their plant or insect origin of isolation are all able to colonize plant and insect hosts, to cause lethal infections in a Lepidopteran insect and to provide protection against a soilborne plant pathogen.

Very often, phylogeny does not correspond with phenotype, especially when there are not so many phenotypically characterized strains in a particular taxonomical group [73] which is the case for insecticidal pseudomonads. In our study, the pathogenicity of the new *P. chlororaphis* isolates does not correlate with their position within the clusters of the *P. chlororaphis* phylogenetic tree (Fig. 1). Moreover, six very closely related strains not distinguishable from each other in the phylogenetic tree based on *gyrB*/*rpoD*/*rpoB* genes display significant differences in oral insecticidal activity and insect colonization abilities (Figs. 1 - 3, Supplementary Figures 2 and 3, Table 2, Supplementary Tables S3 and S4) .We speculate that these phenotypic differences could be related to small genetic and regulatory variations as shown for antimicrobial resistance in *P. aeruginosa* [4], or other genomic plasticity mechanisms of importance for bacterial adaptation [2, 74].

### 4.4. Small genetic mutations can explain phenotypic differences.

Small genetic changes can lead to phenotypic differences in phylogenetically close strains. We found phenotypic differences in our insect feeding assays where the Coleoptera isolates PCLAR01 and PCLAR04 were delayed in killing compare to the Diptera isolate PCLAR03 or the root strain PCL1391 (Fig. 2). Therefore, we performed a single nucleotide polymorphism (SNP) analysis comparing the insect isolates PCLAR01, PCLAR03 and PCLAR04 with the closely related root isolate PCL1391. We found that the total SNPs were homogeneously distributed across the genome of the three strains (Supplementary Figure S6). SNPs differed between the three strains but a principal component analysis (PCA) of these small genetic changes revealed that Coleoptera isolates PCLAR01 and PCLAR04 were more similar to each other than to the Diptera isolate PCLAR03 (Supplementary Figure S7) which reflects the phenotypic data. In addition, the Coleoptera strains were isolated in different years (Table 1) which might be an indication that PCLAR04 is a descendant strain of PCLAR01 able to colonize Coleoptera species. Both strains were isolated from healthy Coleoptera (*Aphodinae* and *Scarabeide* genus respectively) but not from the soil. We hypothesize that these strains might be commensals of the Coleoptera order but further studies investigating the relationship of these two isolates with different Coleoptera and other insects would be needed to confirm this assumption.

PCLAR01 and PCLAR04 has ~25000 mutations and PCLAR03 ~29000 compared to the PCL1391 genome but only between 15 and 19% of the total changes were intragenic non-synonymous mutations i.e. cause an amino acid- or structural change in the resulting protein (Table 3). PCLAR03 harbours regions without any non-synonymous mutations while the same regions in PCLAR01 and PCLAR04 showed higher genetic variability (Fig. 6). The changes in those specific regions might be the reason why the Coleoptera strains are delayed in insect killing. It has been shown in *Yersinia pestis* and *Salmonella enterica* serovar typhi that, in order

to adapt to a specific host, it is necessary to inactivate certain coding regions of the genome [75, 76]. On the other hand, intergenic modifications could result in important changes in regulatory sequences such as small RNAs, riboswitches, promoters, terminators and regulatory binding sites [77]. These non-coding regions were shown to be under selective pressure in several pathogenic bacterial species e.g. *Escherichia coli*, *Klebsiella pneumoniae*, *S. enterica*, *Staphylococcus aureus* and *S. pneumoniae* [78, 79].

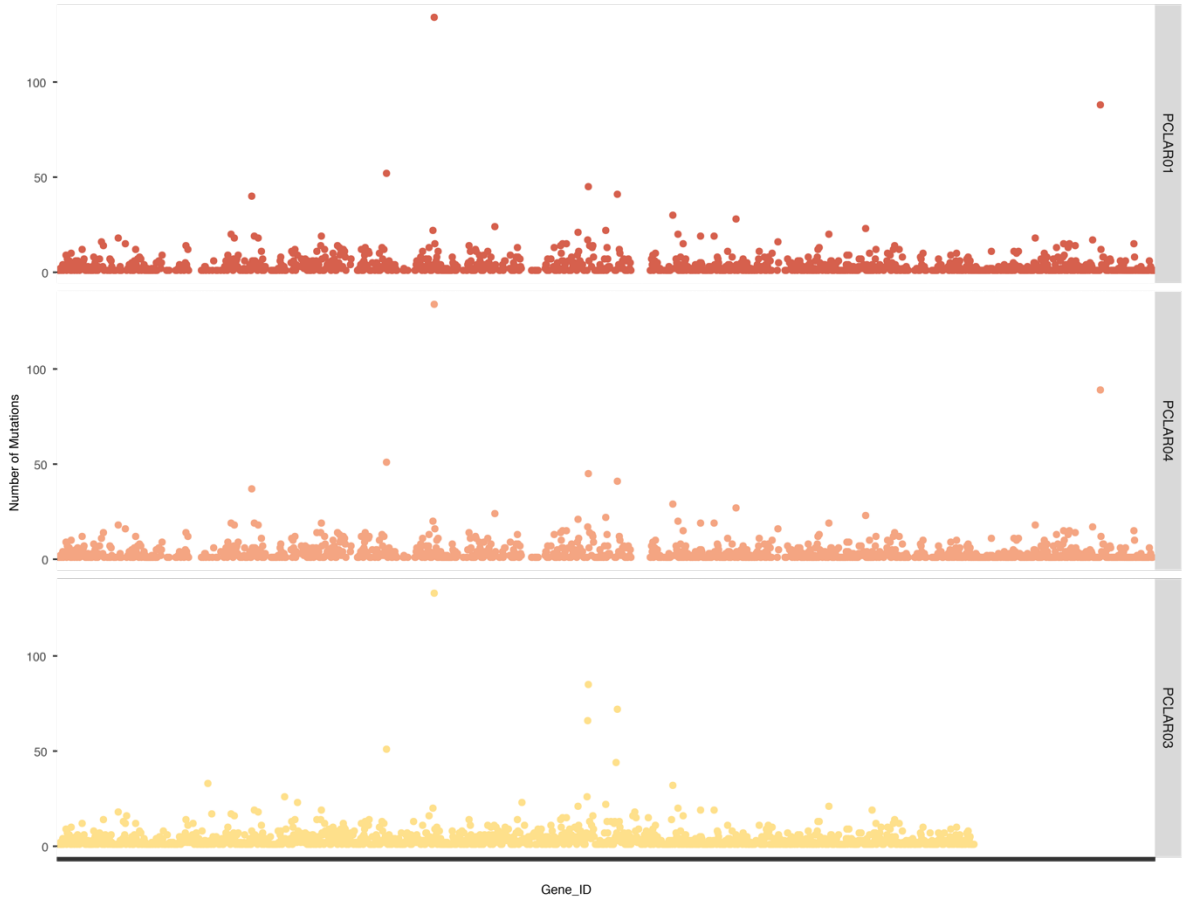


**Figure 6: Number of non-synonymous mutations per gene in *P. chlororaphis* PCLAR01, PCLAR04 and PCLAR03 compared to *P. chlororaphis* PCL1391**. Strains were isolated from roots (*P. chlororaphis* PCL1391), from Coleoptera (*P. chlororaphis* PCLAR01 and *P. chlororaphis* PCLAR04) or Diptera (*P. chlororaphis* PCLAR03).

**Table 3: Total mutations in the genomes of *P. chlororaphis* PCLAR01, PCLAR04 and PCLAR03 isolated from insects compared to *P. chlororaphis* PCL1391.** Intergenic mutations affect non-coding sequences; synonymous are those changes in the genome that will not modify the amino acids in the protein; non-synonymous will cause a change in amino acid in the protein or generate a stop codon; other changes include in-frame and conservative modifications.

| | | Intragenic | | | |
|---|---|---|---|---|---|
| Strain | Intergenic | Synonymous | Non-synonymous | Other | Total |
| PCLAR01 | 2710 | 18087 | 4092 | 885 | 25774 |
| PCLAR03 | 2969 | 20881 | 5436 | 77 | 29363 |
| PCLAR04 | 2748 | 18127 | 4907 | 77 | 25859 |

In our study, we examined, in more detail, the SNPs affecting the insecticidal factors *chiD*, *fitD*, *plcN* [38, 45] and also genes encoding effector proteins of two-partner secretion (TPS) systems (thesis chapter 3). The Coleopteran isolates PCLAR01 and PCLAR04 showed more mutations than PCLAR03 which might be an indication of selective pressure in these genes.

The *fitD* gene showed SNPs in all three strains but only few mutations affected the TcdA-TcdB pore forming domain, which is the active domain of the protein. PCLAR01 and PCLAR04 exhibits five changes in amino acids while PCLAR03 only had two. Of those, PCLAR04 and PCLAR01 showed four changes in type of aminoacid and PCLAR03 only one. Additionally, PCLAR03 has a SNP that could lead to a stop codon upstream the active domain but the insecticidal activity of this strain was even enhanced in one experiment. This might lead to a shorter version of the Fit toxin that seems not to affect the virulence of the bacterium. The *chiD* gene only had one out of two mutations leading to a change of type of amino acid and only in the Coleoptera isolates. Finally, the *plcN* gene showed in all three isolates only one change from serine to proline, which are both polar non-charged aminoacids, therefore, the SNP might not have a functional effect (Fig. 7).

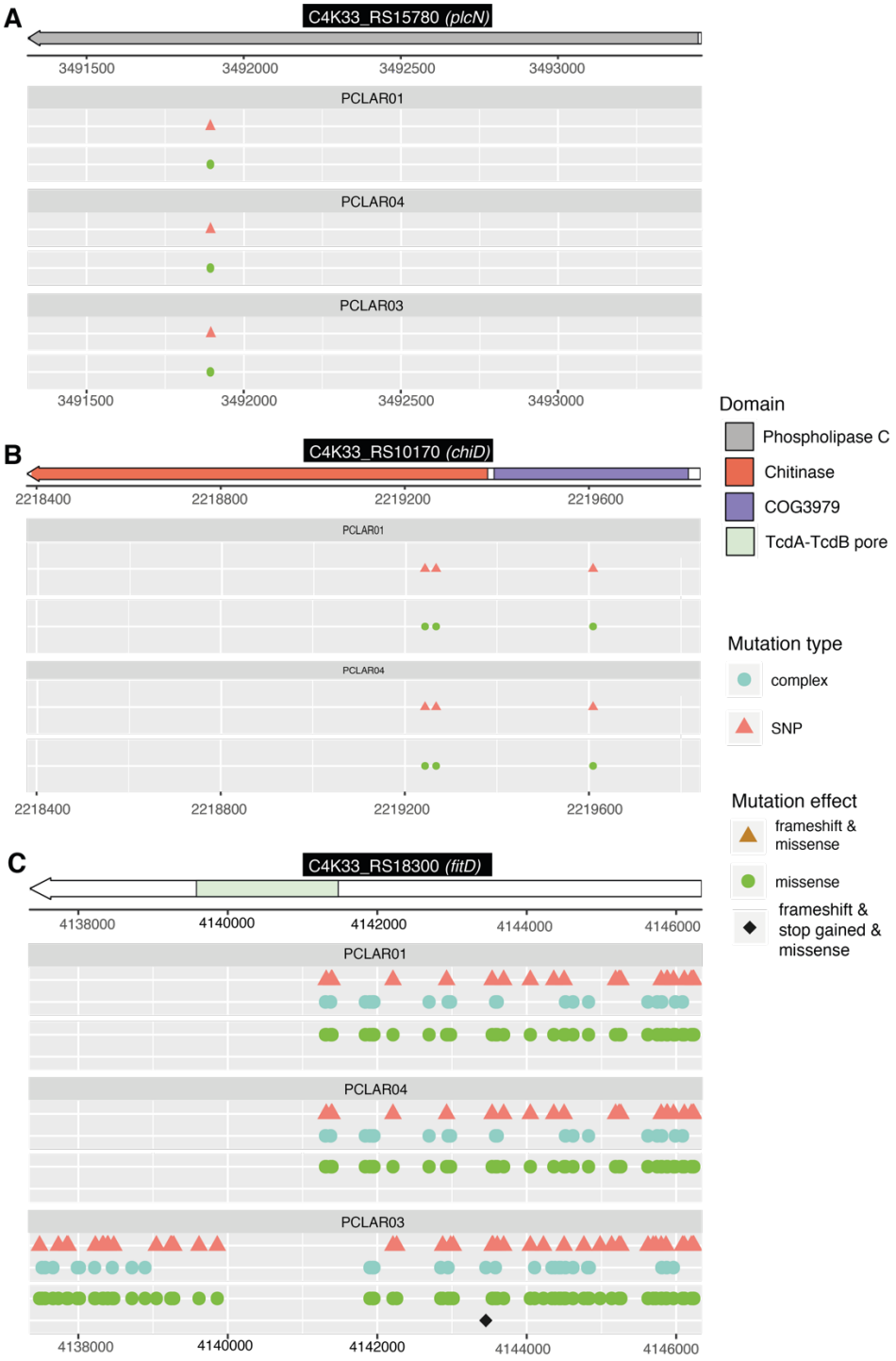


**Figure 7: Non-synonymous mutations in the hemagglutinine-like coding sequences of the phospholipase *plcN* (A), the chitinase *chiD* (B) and the insecticidal toxin *fitD* (C).** Gene sequences are shown on top of the subpanels and the protein-coding domains within sequences are indicated. Lower panels show the mutations harboured by strains *P. chlororaphis* PCLAR01 (Coleoptera isolate), PCLAR04 (Coleoptera isolate) and PCLAR03 (Diptera isolate) compared to the *P. chlororaphis* PCL1391 genome. The phospholipase C domain corresponds to a non-hemolytic phospholipase; the chitinase domain has the active function of degrading chitin; COG 3979 is a chitodextrinase domain that catalizes hydrolysis of chitin oligosaccharides; the TcdA-TcdB domain of the *fitD* toxin forms a pore in the target cell (Ruffner et al., 2015). Mutation type: SNP = single nucleotide polymorphisms; complex = insertions, deletions or larger sequence changes. Mutation effect: modifications that the mutations will cause in the protein i.e. missense when they cause a change of amino acid and frame-shift when the translation frame is affected.

In addition to the insecticidal factors described above, we took a closer look at SNPs in three genes homologous to *tpsA* genes encoding effector proteins of two-partner secretion (TPS) systems. C4K33_RS19905 and C4K33_RS21280 have 77.97%, and 80.08% nucleotide identity with the *tpsA2* and *tpsA4* of *P. protegens* CHA0, respectively which have been demonstrated to contribute to insect invasion and killing in this strain (thesis chapter 3, [48]). CK33_ RSA08790 has 80.13% nucleotide identity with *tpsA1* and 80.6% with *tpsA3* of CHA0. We therefore use CHA0 annotation for these genes and will further refer to C4K33_RS19905 as *tpsA2*, to C4K33_RS21280 as *tpsA4* and to CK33_ RSA08790 as *tpsA1/A3*.

We observed that compared to PCL1391 *tpsA4* did not have SNPs in any of the analysed strains and PCLAR03 did not show any changes in the *tpsA2* either (Supplementary Table S5). On the other hand, the two Coleoptera isolates PCLAR01 and PCLAR04 displayed many missense variations in *tpsA2* encoding the TpsA2 effector i.e. mutations that lead to a predicted amino acid change in the protein (Fig. 8). We speculate that these mutations in *tpsA2* detected in the two Coleoptera isolates could lead to reduced, respectively, slowed down oral activity observed for these isolates in the *Plutella* feeding assays. In *P. protegens* CHA0 *tpsA2* is suggested to be involved in insect gut colonization/transmigration from gut to hemolymph.

The three *P. chlororaphis* strains show, compared to PCL1391 more synonymous than non-synonymous mutations for all these insecticidal factors i.e. *fitD*, *chiD*, *plcN* and *tpsA2* (Supplementary Table 5). This means that the deleterious mutations would probably disappear in the bacterial lineage. This is not the case for the third analysed TPSA encoding gene *tpsA1/A3*, which exhibits more non-synonymous than synonymous mutations (Supplementary Table 5). This gene shows a high concentration of missense mutations throughout the gene in the two Coleoptera isolates, but only at the 5' end of the gene for the Diptera isolates (Fig. 8). This might indicate that this gene is under positive selection generating genomic changes allowing the strain to evade the host-immune response. TPSA encoding genes have been

related to insecticidal activity in *P. protegens* CHA0 (thesis chapter 3), and macrophage killing and pathogenesis in *P. aeruginosa* PA7 and *Serratia marcescens* [80, 81]. Therefore, the diversification of TPSA proteins might be important to establish a functional host-pathogen interaction and the protein versions of PCLAR01 and PCLAR04 might be defective due to the different mutations in the nucleotide sequence.

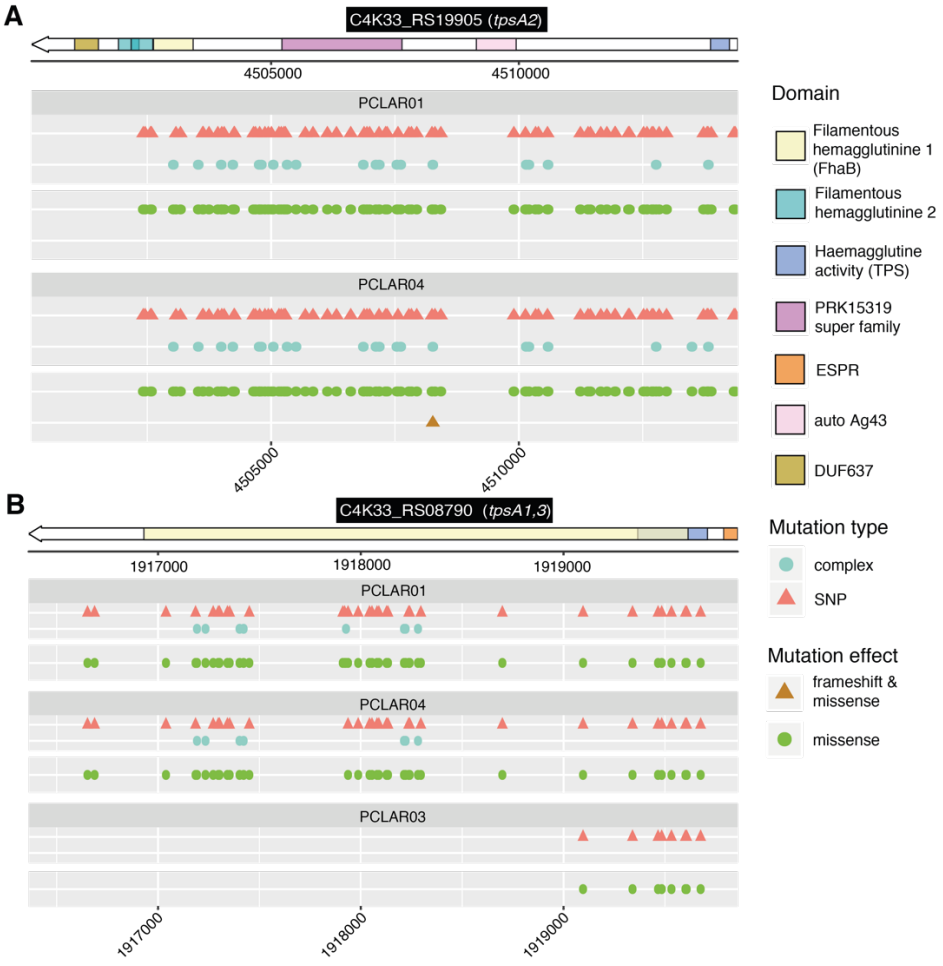


**Figure 8: Non-synonymous mutations in the putative two-partner secretion (TPS) coding sequences C4K33_RS19905 (*tpsA2*) (A) and C4K33_RS08790 (*tpsA1,3*) (B).** Gene sequences are shown on top of the subpanels and the protein-coding domains within sequences are indicated. Lower panels show the mutations harboured by strains *P. chlororaphis* PCLAR01 (Coleoptera isolate), PCLAR04 (Coleoptera isolate) and PCLAR03 (Diptera isolate) compared to the *P. chlororaphis* PCL1391 genome. The haemagglutinine activity (TPS) domain are used to interact with the transporter protein for membrane translocation; the filamentous haemagglutinines are repeats used to attach to the host cells and translocate into the host; Filamentoud hemagglutinine 1 (FhaB): filamentous-haemagglutinine domain of *Bordetella pertussis*; PRK15319: fibronectin-binding autotransporter adhesin type SdhA; ESPR: signal domain related to type V secretion systems; Ag43: autotransporter related to type V secretion systems; DUF637: domain of unknown function, which appears associated with TPS proteins (Reboud et al., 2017-review; Allen and Hausser, 2017). Mutation type: SNP = single nucleotide polymorphisms; complex = insertions, deletions or larger sequence changes. Mutation effect: modifications that the mutations will cause in the protein i.e. missense when they cause a change of amino acid and frame-shift when the translation frame is affected.

High genetic variation allows the bacterial population to rapidly expand and fill a new niche or colonize a new host. The easy switch of hosts as it seems to be the case for *P. protegens* and *P. chlororaphis* indicates that these bacteria have developed as generalists rather than specialists. The capability of a bacterium to colonize a new host depends on its ability to easily adapt to new environments [82, 83]. *Pseudomonas* undergo numerous genetic re-arrangements during host colonization as a result of the phase variation process, which increases the variability of the population and allows it to survive in a new environment [84]. Therefore, it might be interesting to study host-specific genomics in *Pseudomonas* in the future to see if the bacterial inhabitants of different arthropods harbour specific mutations depending on the host organism or the isolation site. This will give us new insights into the development of specialistic or rather generalist variants within the *Pseudomonas* genus.

## 5. Conclusion

*Pseudomonas* are present in very different environments as free bacteria but also associated to other organisms in pathogenic, commensal or beneficial relationships. Our study shows for the first time that insecticidal pseudomonads belonging to the two species *P. protegens* and *P. chlororaphis* are natural inhabitants of several insect and myriapod classes. Interestingly all *P. protegens* strains isolated in this study were always associated with insects meanwhile we found insecticidal *P. chlororaphis* associated with insects, myriapods, roots and soil which is an indication for the variability and adaptability of this species. All *P. protegens/chlororaphis* strains we have characterized so far either in this or in earlier studies [45] have the ability to colonize plants and insect hosts, insecticidal activity and control root pathogens. Our results presented here show that *P. protegens* isolates are much more consistent in insecticidal abilities meanwhile *P. chlororaphis* were more variable which might indicate a certain adaptation to different hosts. However, this remains purely speculative since also all *P. protegens* isolates

obtained in this study, although highly lethal to *P. xylostella*, were isolated from healthy insects. Altogether, our observations raise the question whether these species are evolving towards commensal, pathogenic and symbiotic interactions with arthropods as a new niche or if they are just very good colonizers able to adapt to any new environment. This exposes how far we are of fully understand their ecology and the particular mechanisms allowing them to colonize such different environments. We observed that isolation site or phylogeny does not always resemble the insecticidal capabilities of the fluorescent *Pseudomonas* as closely related bacteria showed differential insecticidal activity. In two *P. chlororaphis* insect isolates displaying lower insecticidal activity we have identified non-synonymous mutations in some insecticidal factors which could explain their reduced or slowed down insect killing capacities. Our findings corroborate that these fascinating bacteria are multi-talented and able to conquer very different niches and exploit cross kingdom hosts. The ability of *P. protegens* and *P. chlororaphis* to successfully colonize plant roots, enhance plant growth and control the raise of external menaces, such as root pathogens or insect pests, makes them suitable candidates for future biocontrol applications.

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# 8. Supplementary material

## 8.1. Supplementary figures

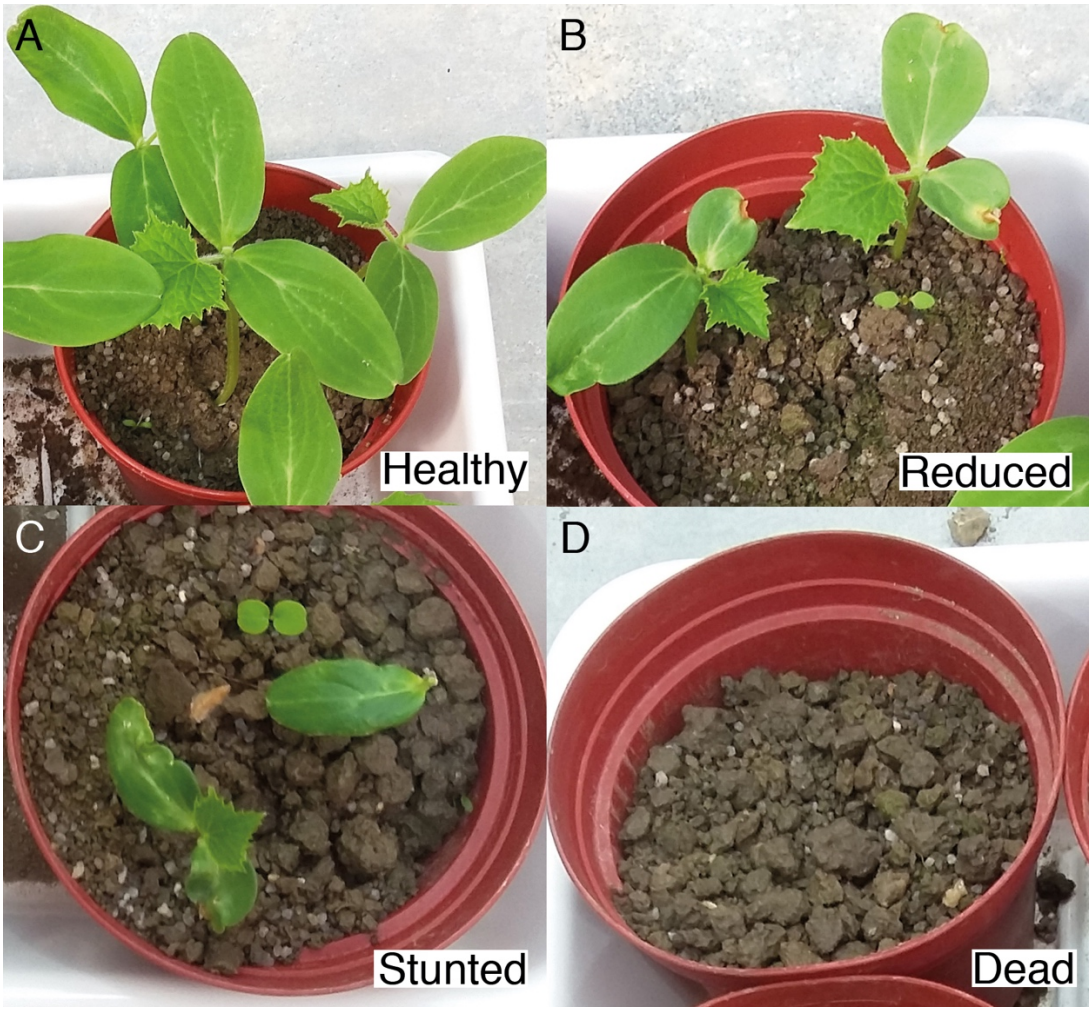


**Figure S1: Disease classfication for cucumber plants infected with the oomycete pathogen *Pythium ultimum*.** Cucumber seeds of the variety Chinese Snake were inoculated with 0 g or 1 g of *P. ultimum*. Disease was classified as A) healthy, B) reduced growth, C) stunted plants and D) dead plants.

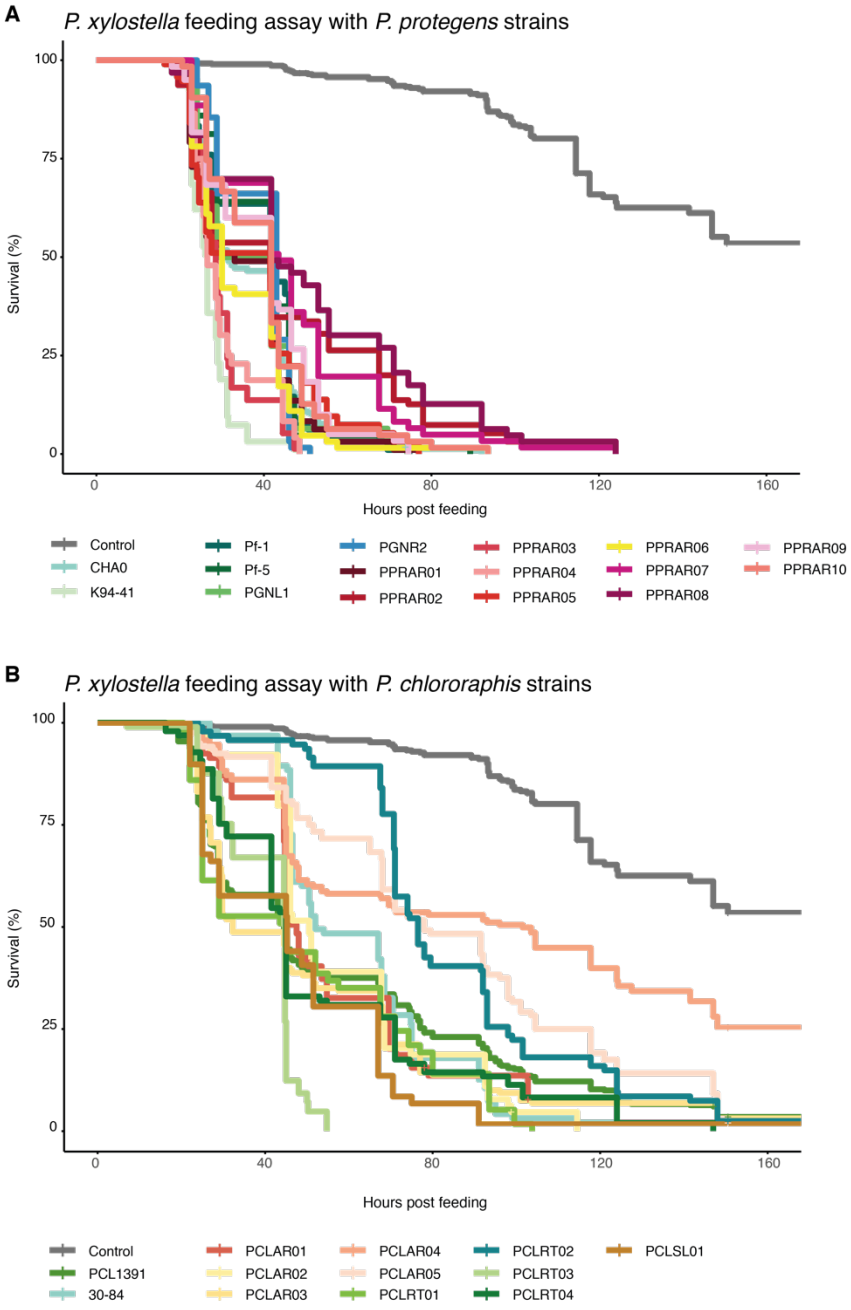


**Figure S2: Survival of *Plutella xylostella* larvae after oral uptake of different *Pseudomonas protegens* (A) and P*seudomonas chlororaphis* (B) strains.** Second instar *P. xylostella* larvae were fed with artificial diet pellets spiked with 4 x $10^6$ bacterial cells and mortality was assessed periodically by poking the insects. Strains were isolated from roots or from arthropods. Data from 16 different experiments were pooled including the experiment as an extra factor. Survival curves of individual experiments are shown in Supplementary Figure S3 and associated statistics (*p* values of log rank tests) in Supplementary Table S4.

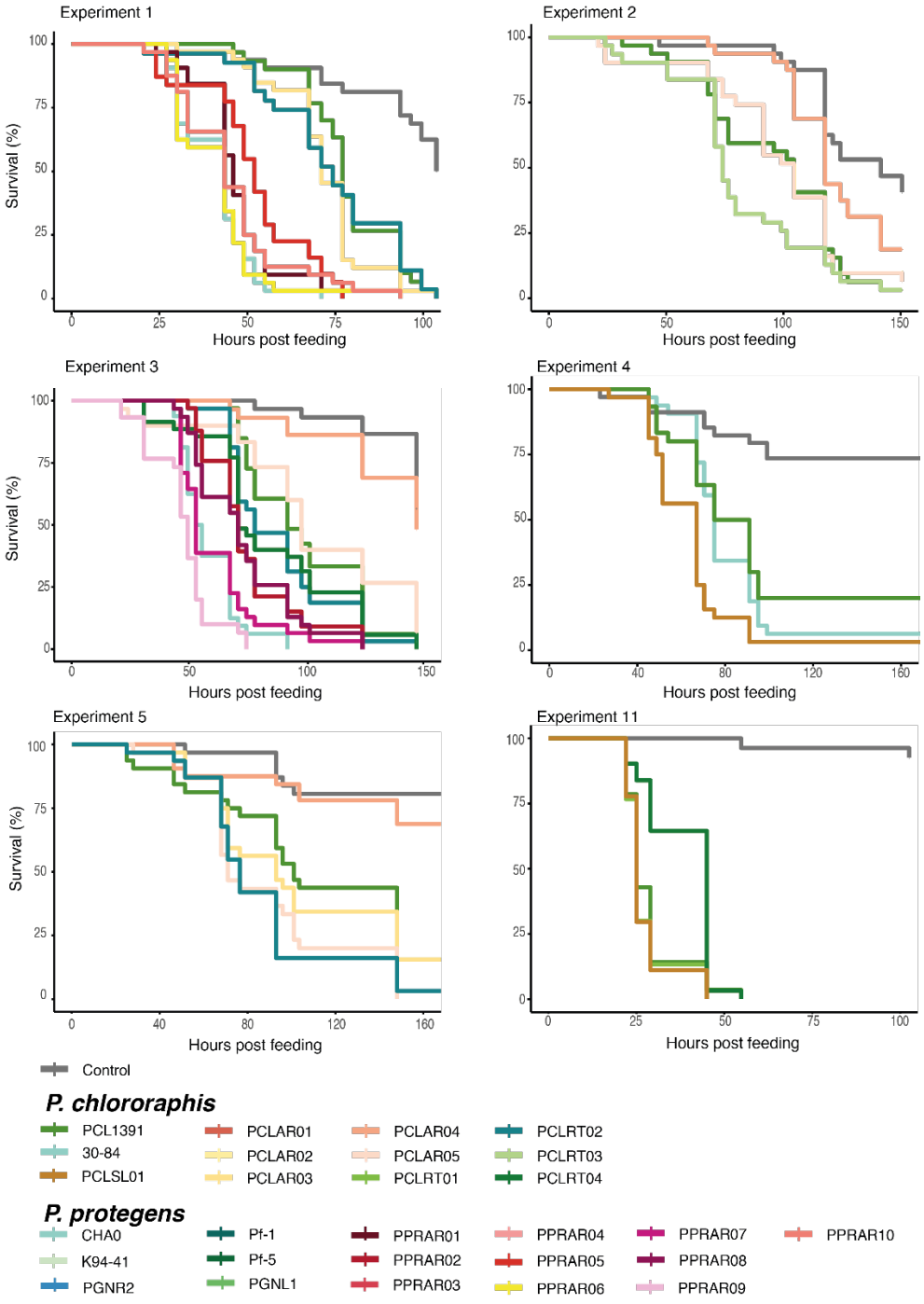


**Figure S3: Kaplan Meyer graphs showing survival of *Plutella xylostella* larvae in feeding assays with different *Pseudomonas protegens* and *Pseudomonas chlororaphis* strains.** Thirty-two second instar larvae *P. xylostella* were fed with artificial diet pellets spiked with 4 x $10^6$ bacterial cells and mortality was assessed periodically by poking the insects. Statistically significant differences between strains were assessed using the log rank test of the "survival" package in R. *p* values are shown in Supplementary Table S4.

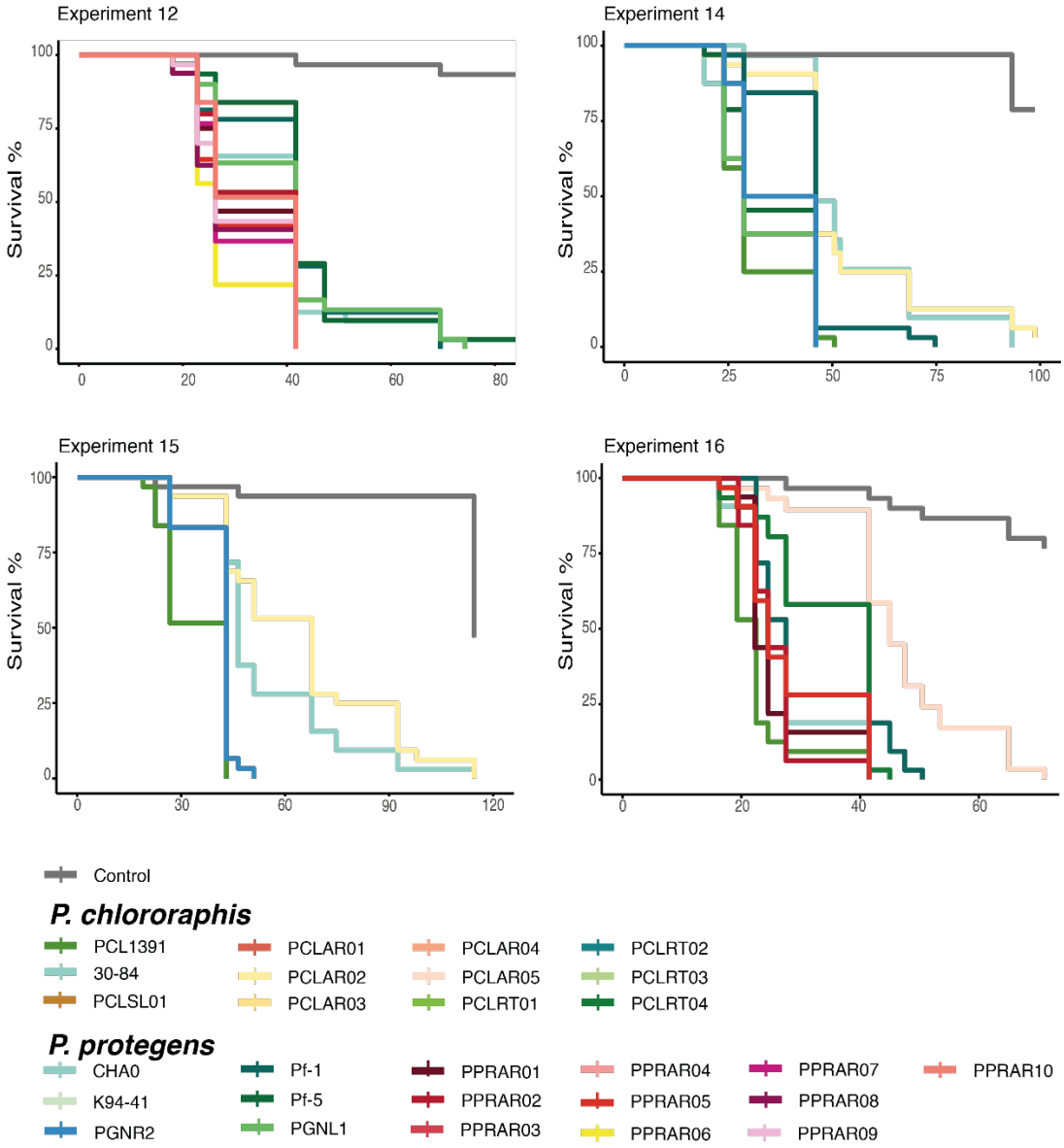


**Figure S3 continued: Kaplan Meyer graphs showing survival of *Plutella xylostella* larvae in feeding assays with different *Pseudomonas protegens* and *Pseudomonas chlororaphis* strains.** Thirty-two second instar larvae *P. xylostella* were fed with artificial diet pellets spiked with 4 x $10^6$ bacterial cells and mortality was assessed periodically by poking the insects. Statistically significant differences between strains were assessed using the log rank test of the "survival" package in R. *p* values are shown in Supplementary Table S4.

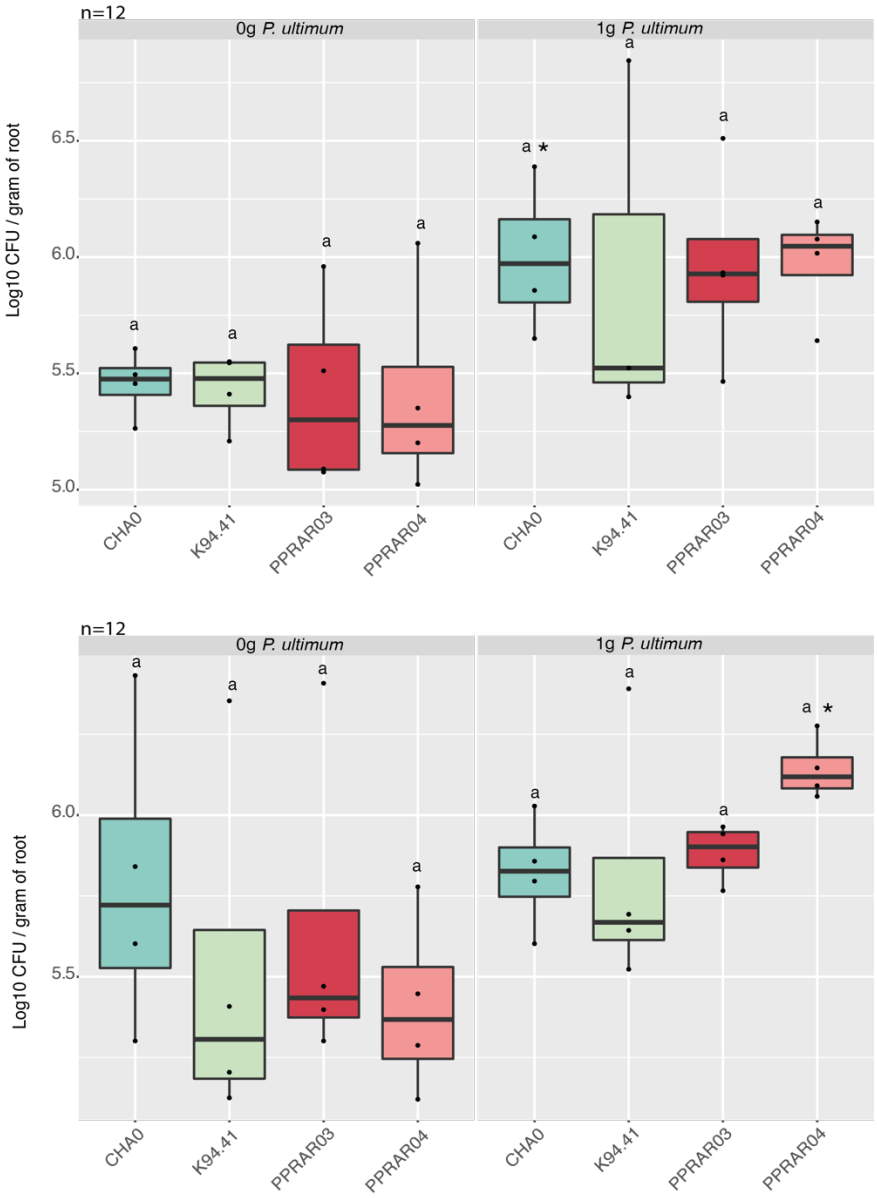


**Figure S4: Colonization of healthy (0 g) and *P. ultimum*-infected (1 g) cucumber roots by *P. protegens* root and arthropod isolates.** Two independent experiments are shown. Strains were isolated from roots (strains *P. protegens* CHA0 and *P. protegens* K94.41) or from arthropods (strains *P. protegens* PPRAR03 and *P. protegens* PPRAR04). Boxplots for the same pathogen concentration marked with the same letters do not significantly differ from each other (Dunn's test $p$-value<0.05). Those strains for which root colonization was significantly different in presence of 1 g of *P. ultimum* compared to the non-infected control are marked with and asterisk (Kruskal-Wallis test $p$-value<0.05).

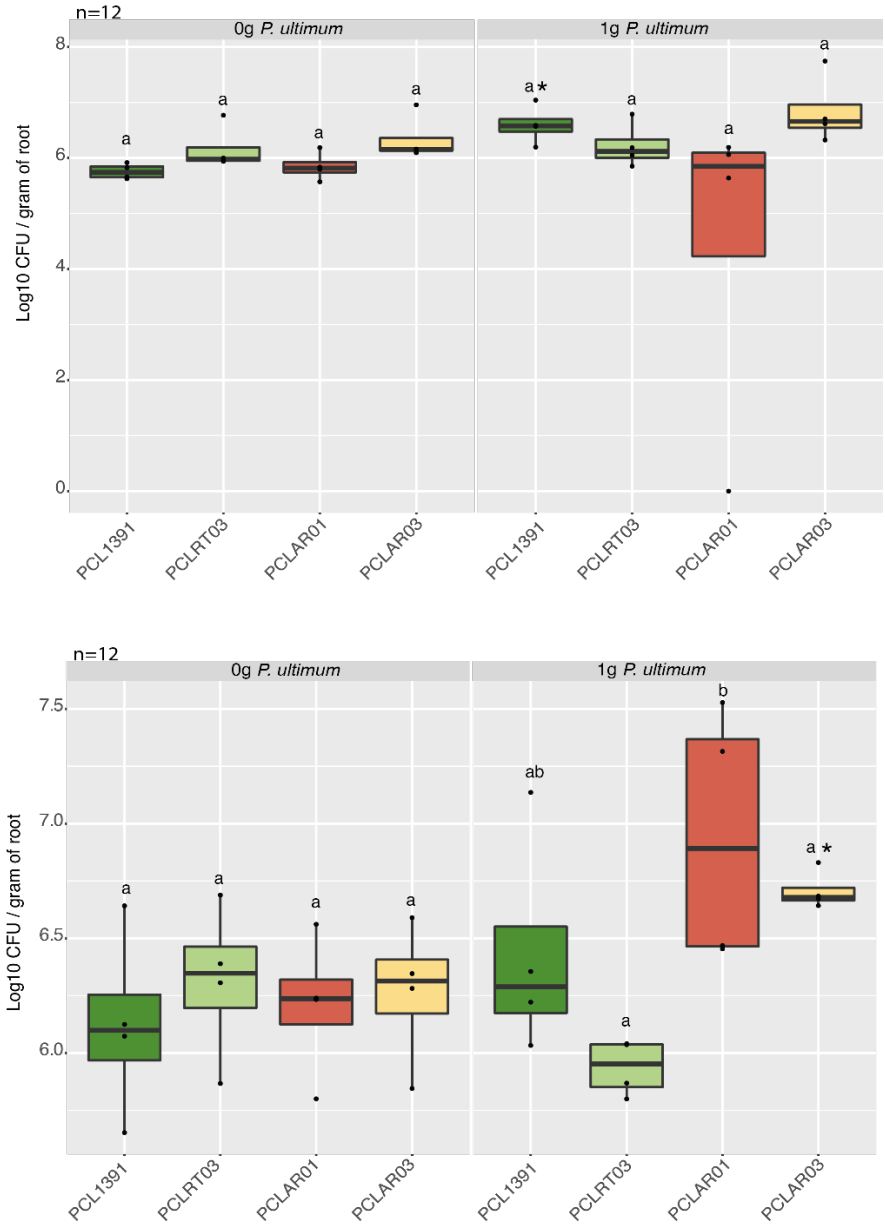


**Figure S5: Colonization of healthy (0 g) and *P. ultimum*-infected (1 g) cucumber roots by *P. chlororaphis* root and arthropod isolates.** Two independent experiments are shown. Strains were isolated from roots (strains *P. chlororaphis* PCL1391 and *P. chlororaphis* PCLRT03) or from arthropods (strains *P. chlororaphis* PCLAR01 and *P. chlororaphis* PCLAR03). Boxplots for the same pathogen concentration marked with different letters are significantly different (Dunn's test $p$-value<0.05). Those strains for which root colonization was significantly different in presence of 1 g of *P. ultimum* compared to the non-infected control are marked with and asterisk (Kruskal-Wallis test $p$-value<0.05).

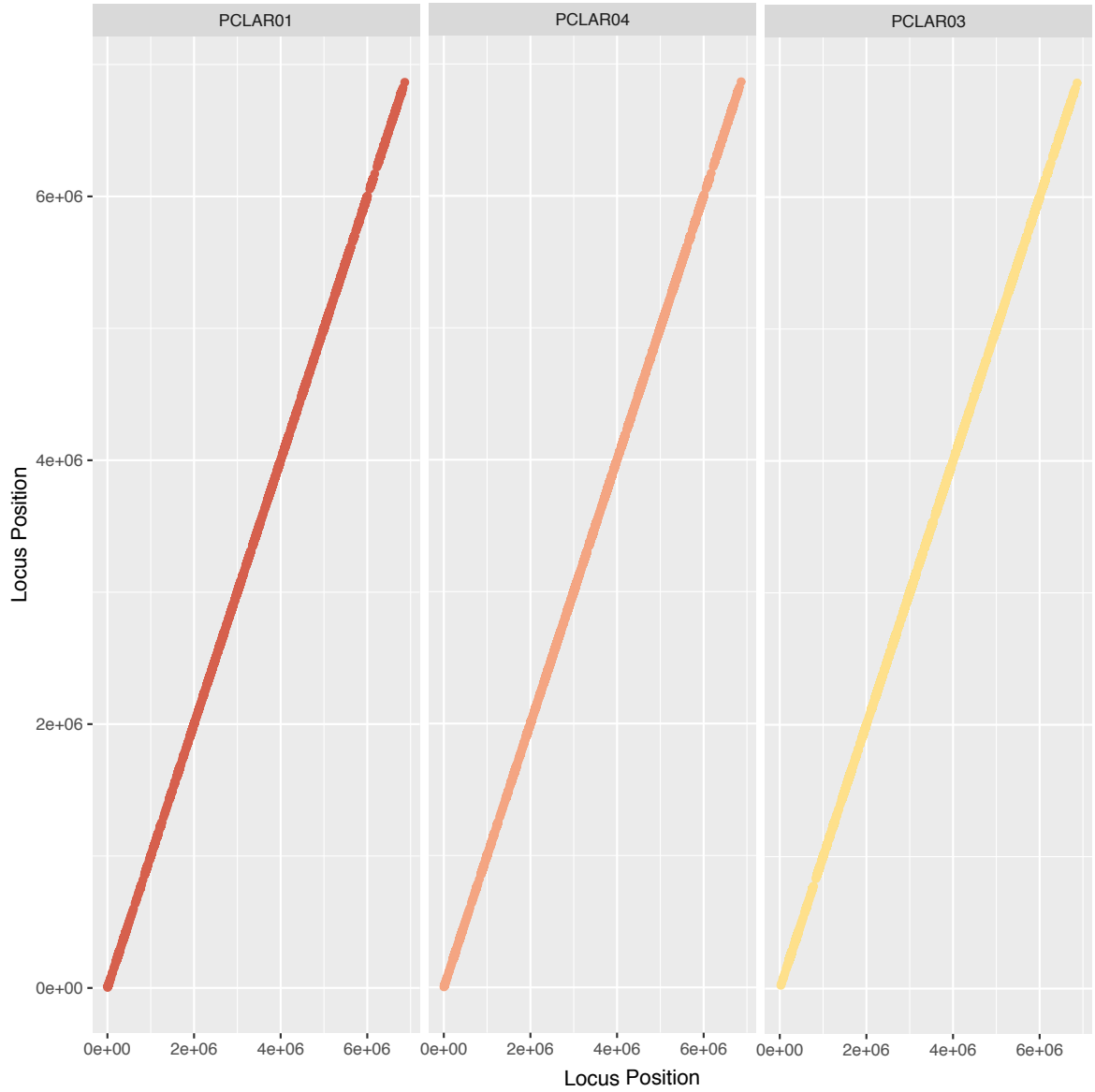


**Figure S6: Distribution of mutations across the genomes of *P. chlororaphis* PCLAR01, PCLAR04 and PCLAR03.** *P. chlororaphis* PCL1391 was used as reference genome in a SNP calling analysis of the *P. chlororaphis* strains PCLAR01, PCLAR04 and PCLAR03 strains. All loci displaying a mutation compared to the PCL1391 reference genome are depicted.

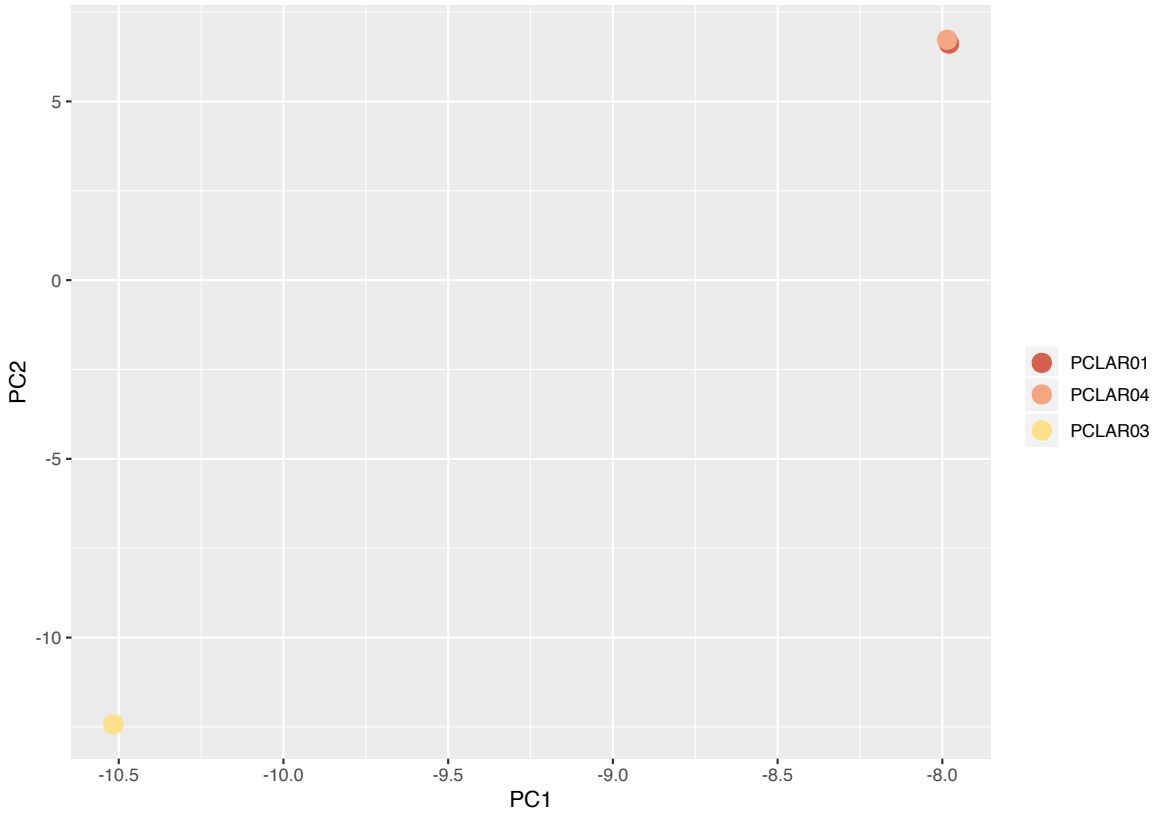


**Figure S7: Principal component analysis (PCA) analysis of the genome polymorphisms of *P. chlororaphis* strains PCLAR01, PCLAR04 and PCLAR03 strains compared to *P. chlororaphis* PCL1391.** The analysis is based on a total of 80'996 genome-wide SNP genotypes.

## 8.2. Supplementary tables

**Table S1. List of *Pseudomonas* genomes used for the tree depicted in Figure 1.**

| Strain | Isoltation | Assembly | Reference |
|---|---|---|---|
| *P. protegens* CHA0 | Plant | GCF_900560965.1_PPRCHA0 | [1] |
| *P. protegens* PGNR1 | Plant | GCF_001269475.1_ASM126947v1 | [2] |
| *P. protegens* Pf-1 | Plant | | This study |
| *P. protegens* PGNL1 | Plant | | This study |
| *P. protegens* PGNR2 | Plant | | This study |
| *P. protegens* BRIP | Arthropod | GCF_001269495.1_ASM126949v1 | [3] |
| *P. protegens* K94.41 | Plant | GCF_001269485.1_ASM126948v1 | [4] |
| *P. protegens* PPRAR10 | Arthropod | | This study |
| *P. protegens* PPRAR02 | Arthropod | | This study |
| *P. protegens* PPRAR01 | Arthropod | | This study |
| *P. protegens* PPRAR03 | Arthropod | | This study |
| *P. protegens* PPRAR04 | Arthropod | | This study |
| *P. protegens* PPRAR05 | Arthropod | | This study |
| *P. protegens* PPRAR06 | Arthropod | | This study |
| *P. protegens* PPRAR07 | Arthropod | | This study |
| *P. protegens* PPRAR08 | Arthropod | | This study |
| *P. protegens* PPRAR09 | Arthropod | | This study |
| *P. protegens* Pf.5 | Plant | GCF_000012265.1_ASM1226v1 | [5] |
| *P. protegens* PF | Plant | GCF_001269465.1_ASM126946v1 | [6] |
| *Pseudomonas* sp. CMR12a | Plant | GCF_003850565.1_ASM385056v1 | [7] |
| *Pseudomonas*_sp. CMR5c | Plant | GCF_003850545.1_ASM385054v1 | [7] |
| *P. chlororaphis* subsp. *Aureofaciens* CD | Arthropod | GCF_001269595.1_ASM126959v1 | [3] |
| *P. chlororaphis* PCLRT02 | Plant | | This study |
| *P. chlororaphis* PCLAR05 | Arthropod | | This study |
| *P. chlororaphis* subsp. *aureofaciens* LMG 1245 | Other | GCF_001269575.1_ASM126957v1 | [8] |
| *P. chlororaphis* subsp. *piscium* DSM 21509 | Other | GCF_003850345.1_ASM385034v1 | [9] |
| *P. chlororaphis* PCLRT04 | Plant | | This study |
| *P. chlororaphis* PCLRT03 | Plant | | This study |
| *P. chlororaphis* subsp. *aureofaciens* 30.84 | Plant | GCF_000281915.1_ASM28191v1 | [10] |
| *P. chlororaphis* subsp. Chlororaphis DSM 50083 | Other | GCF_003945765.1_ASM394576v1 | [11] |
| *P. chlororaphis* PCLAR03 | Arthropod | | This study |
| *P. chlororaphis* PCLAR01 | Arthropod | | This study |
| *P. chlororaphis* PCLAR04 | Arthropod | | This study |
| *P. chlororaphis* PCLAR02 | Arthropod | | This study |
| *P. chlororaphis* PCLRT01 | Arthropod | | This study |
| *P. chlororaphis* PCLSL01 | Other | | This study |
| *P. chlororaphis* subsp. *piscium* PCL1391 | Plant | GCF_003850445.1_ASM385044v1 | [12] |

| | | | |
|---|---|---|---|
| *P. brassicacearum* TM1A3 | Plant | GCF_001269635.1_ASM126963v1 | [2] |
| *P. kilonensis* P12 | Plant | GCF_001269725.1_ASM126972v1 | [2] |
| *P. kilonensis* DSM 13647 | Plant | GCF_001269885.1_ASM126988v1 | [13] |
| *P. thivervalensis* DSM 13194 | Plant | GCF_001269655.1_ASM126965v1 | [14] |
| *P. thivervalensis* PITR2 | Plant | GCF_001269685.1_ASM126968v1 | [2] |
| *Pseudomonas* sp. Q12.87 | Plant | GCF_001269755.1_ASM126975v1 | [2] |
| *Pseudomonas* sp. Pf153 | Plant | GCF_001269775.1_ASM126977v1 | [15] |
| *Pseudomonas* sp. P97.38 | Plant | GCF_001269745.1_ASM126974v1 | [4] |
| *Pseudomonas* sp. P1.31 | Plant | GCF_001269815.1_ASM126981v1 | [3] |
| *P. fluorescens* DSM 50090 | Plant | GCF_001269845.1_ASM126984v1 | [16] |
| *P. lactis* SS101 | Plant | GCF_000263675.1_ASM26367v2 | [17] |
| *Pseudomonas* sp. MIACH | Plant | GCF_001269925.1_ASM126992v1 | [18] |
| *Pseudomonas* sp. P1.8 | Plant | GCF_001269805.1_ASM126980v1 | [3] |
| *P. gingeri* NCPPB 3146 | Other | GCF_002895165.1 | [19] |

## Table S2 characteristics of the genomes

| | | | | | Tetra | | ANIm | |
|---|---|---|---|---|---|---|---|---|
| **Species** | **Strain** | **DNA extraction method** | **Seq. Tecnology** | **%GC** | **P.chlororaphis PCL1391** | **P.protegens CHA0** | **P.chlororaphis PCL1391** | **P.protegens CHA0** |
| *P. chlororaphis* | PCL1391 | | | 62.8 | --- | 0.98748 | --- | 87.34 |
| *P. chlororaphis* | 30-84 | | | 62.9 | 0.99933 | 0.98802 | 94.81 | 87.39 |
| *P. chlororaphis* | PCLAR01 | Promega | MiSeq | 61.7 | 0.99979 | 0.98746 | 99.17 | 87.4 |
| *P. chlororaphis* | PCLAR02 | Promega | MiSeq | 59.82 | 0.99971 | 0.98718 | 99.15 | 87.45 |
| *P. chlororaphis* | PCLAR03 | Promega | MISeq | 58.6 | 0.99975 | 0.98679 | 99.08 | 87.5 |
| *P. chlororaphis* | PCLAR04 | Qiagen | PacBio | 62.6 | 0.99984 | 0.98764 | 99.28 | 87.35 |
| *P. chlororaphis* | PCLAR05 | Qiagen | PacBio | 62.99 | 0.99931 | 0.98768 | 95.77 | 87.44 |
| *P. chlororaphis* | PCLRT01 | Promega | MiSeq | 63.3 | 0.99999 | 0.98748 | 99.84 | 87.34 |
| *P. chlororaphis* | PCLRT02 | Qiagen | PacBio | 63.1 | 0.99932 | 0.98767 | 95.98 | 87.53 |
| *P. chlororaphis* | PCLRT03 | Promega | MiSeq | 59.06 | 0.99976 | 0.98631 | 96.61 | 87.4 |
| *P. chlororaphis* | PCLRT04 | Qiagen | PacBio | 62.6 | 0.99978 | 0.98674 | 96.74 | 87.26 |
| *P. chlororaphis* | PCLSL01 | Qiagen | PacBio | 62.7 | 0.99993 | 0.98717 | 99.19 | 87.35 |
| | | | | | | | | |
| *P. protegens* | CHA0 | | | 63.4 | 0.98748 | --- | 87.35 | --- |
| *P. protegens* | K94-41 | | | 61.23 | 0.98768 | 0.99993 | 87.37 | 99 |
| *P. protegens* | Pf-1 | Promega | MiSeq | 61.39 | 0.98755 | 0.99998 | 87.36 | 99.91 |
| *P. protegens* | Pf-5 | | | 63.3 | 0.98716 | 0.9999 | 87.38 | 98.75 |
| *P. protegens* | PGNL1 | Qiagen | PacBio | 63.4 | 0.98748 | 1 | 87.35 | 99.93 |
| *P. protegens* | PGNR2 | Qiagen | PacBio | 63.4 | 0.98748 | 1 | 87.35 | 99.93 |
| *P. protegens* | PPRAR01 | Promega | MiSeq | 60.91 | 0.98779 | 0.99988 | 87.35 | 98.62 |
| *P. protegens* | PPRAR02 | Qiagen | PacBio | 63.3 | 0.98746 | 0.99992 | 87.42 | 98.83 |
| *P. protegens* | PPRAR03 | Promega | MiSeq | 60.59 | 0.98832 | 0.99968 | 87.52 | 98.65 |
| *P. protegens* | PPRAR04 | Promega | MiSeq | 58.92 | 0.98806 | 0.99963 | 87.5 | 98.63 |
| *P. protegens* | PPRAR05 | Promega | MISeq | 58.89 | 0.9884 | 0.99966 | 87.52 | 98.63 |
| *P. protegens* | PPRAR06 | Promega | MiSeq | 58.83 | 0.98746 | 0.99975 | 87.44 | 98.66 |
| *P. protegens* | PPRAR07 | Qiagen | PacBio | 63.3 | 0.98736 | 0.99993 | 87.32 | 98.72 |
| *P. protegens* | PPRAR08 | Qiagen | PacBio | 63.3 | 0.98734 | 0.99993 | 87.33 | 98.75 |
| *P. protegens* | PPRAR09 | Qiagen | PacBio | 63.3 | 0.98732 | 0.99992 | 87.34 | 98.76 |
| *P. protegens* | PPRAR10 | Promega | MISeq | 60.65 | 0.98793 | 0.9998 | 87.42 | 98.64 |

**Table S3. LT50 values from feeding *P. xylostella* with different *P. chlororaphis* and *P. protegens strains*.** Confidence values are depicted between brackets and significances compared to the reference strain are indicated with letters (p-value <0.05).

| Species | Strain | isolation | Experiment 1 | | Experiment 2 | | Experiment 3 | | Experiment 4 | | Experiment 5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. chlororaphis* | PCL1391 | Tomato root | 75.2 (72.7; 77.6) | a | 91.5 (87; 95.8) | a | 92.0 (88.1; 96.2) | a | 80.3 (63.9; 115.5) | a | 98.7 (92; 106.4) | a |
| *P. chlororaphis* | 30.84 | Wheat seed | | | | | | | 74 (71; 77) | a | | |
| *P. chlororaphis* | PCLAR01 | Aphodiinae | | | | | | | | | | |
| *P. chlororaphis* | PCLAR02 | Aphodiinae | | | | | | | | | | |
| *P. chlororaphis* | PCLAR03 | Diptera | 65.7 (64.1; 71.1) | b | | | | | | | 93.6 (86.6; 101.4) | ad |
| *P. chlororaphis* | PCLAR04 | Scarabaeidae pupa | | | 118.2 (114.1; 122.9) | b | 145.6 (131.6; 171.9) | b | | | 190.5 (158.7; 273.0) | b |
| *P. chlororaphis* | PCLAR05 | Scarabaeidae larva | | | 93.6 (85.2; 102.1) | a | 96.6 (89.1; 105.0) | a | | | 78.2 (73.5; 82.8) | d |
| *P. chlororaphis* | PCLIN01 | Lumbricidae | | | 91.7 (86.8 ; 96.8) | a | 124.3 (113.1; 141.7) | b | | | | |
| *P. chlororaphis* | PCLLF01 | Medicago | | | | | | | 85.8 (73.8; 108.7) | a | | |
| *P. chlororaphis* | PCLRT01 | grass root | 70 (66.9; 73.1) | a | | | | | | | | |
| *P. chlororaphis* | PCLRT02 | potato root | | | 74.5 (69.7; 79.0) | c | 80.8 (77.1; 84.5) | d | | | 76.1 (67.3; 89.8) | d |
| *P. chlororaphis* | PCLRT03 | potato root | | | | | | | | | | |
| *P. chlororaphis* | PCLRT04 | potato root | | | | | 77.9 (73.6; 82.2) | d | | | | |
| *P. chlororaphis* | PCLRT05 | grass root | | | | | | | 96.9 (88.1; 107.9) | a | 142.1 (126.3; 166.2) | b |
| *P. chlororaphis* | PCLSL01 | potato soil | | | | | | | 56.9 (53.8; 59.9) | b | | |
| *P. protegens* | CHA0 | Tobacco root | 37.8 (35.9; 39.6) | a | | | 54.7 (52.7; 56.8) | a | | | | |
| *P. protegens* | K94.41 | Cucumber root | | | | | | | | | | |
| *P. protegens* | Pf-1 | Tobacco root | | | | | | | | | | |
| *P. protegens* | Pf5 | soil | | | | | | | | | | |
| *P. protegens* | PGNL1 | Tobacco root | | | | | | | | | | |
| *P. protegens* | PGNR2 | Tobacco root | | | | | | | | | | |
| *P. protegens* | PPRAR01 | Lithobius | 43.5 (41.5; 45.4) | b | | | | | | | | |
| *P. protegens* | PPRAR02 | Lithobius | | | | | 70 (67.2; 72.9) | b | | | | |
| *P. protegens* | PPRAR03 | Lepidoptera larvae | | | | | | | | | | |
| *P. protegens* | PPRAR04 | Agriotes | | | | | | | | | | |
| *P. protegens* | PPRAR05 | Staphylinidae | 48.8 (46.2; 51.3) | c | | | | | | | | |
| *P. protegens* | PPRAR06 | Agriotes | 37.1 (34.8; 39.1) | a | | | | | | | | |
| *P. protegens* | PPRAR07 | Lithobius | | | | | 56.3 (50.1; 61.3) | a | | | | |
| *P. protegens* | PPRAR08 | Geophilidae | | | | | 68.5 (65.6; 71.5) | b | | | | |
| *P. protegens* | PPRAR09 | Curculionidae | | | | | 44.6 (39.7; 48.8) | c | | | | |
| *P. protegens* | PPRAR10 | Agrypnus murinus | 41.9 (39.2; 44.5) | ab | | | | | | | | |

| Species | Strain | isolation | Experiment 6 | | Experiment 7 | | Experiment 8 | | Experiment 9 | | Experiment 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. chlororaphis* | PCL1391 | Tomato root | | | | | 37 (35; 38.9) | a | 27 (25; 28.9) | a | 24.5 (21.9; 25.9) | a |
| *P. chlororaphis* | 30.84 | Wheat seed | | | | | ---- | | | | | |
| *P. chlororaphis* | PCLAR01 | Aphodiinae | | | | | 48.2 (45.2; 51.1) | b | 44.3 (41.3; 48.3) | b | 52.3 (46.8; 57.6) | b |
| *P. chlororaphis* | PCLAR02 | Aphodiinae | | | | | | | | | | |
| *P. chlororaphis* | PCLAR03 | Diptera | | | | | 25.6 (22.8; 27.7) | c | 27.5 (26.6; 28.4) | a | 21.9 (10.8; 24.1) | a |
| *P. chlororaphis* | PCLAR04 | Scarabaeidae pupa | | | | | 45.4 (36.1; 55.4) | a | 39.6 (37.3; 42.2) | bc | 38.9 (32.1; 44.8) | c |
| *P. chlororaphis* | PCLAR05 | Scarabaeidae larva | | | | | | | | | | |
| *P. chlororaphis* | PCLIN01 | Lumbricidae | | | | | | | | | | |
| *P. chlororaphis* | PCLLF01 | Medicago | | | | | | | | | | |
| *P. chlororaphis* | PCLRT01 | grass root | | | | | | | | | | |
| *P. chlororaphis* | PCLRT02 | potato root | | | | | | | | | | |
| *P. chlororaphis* | PCLRT03 | potato root | | | | | 33.7 (25.4; 40.8) | ac | 35.7 (33.3; 38.1) | c | 34.6 (32.1; 36.9) | c |
| *P. chlororaphis* | PCLRT04 | potato root | | | | | | | | | | |
| *P. chlororaphis* | PCLRT05 | grass root | | | | | | | | | | |
| *P. chlororaphis* | PCLSL01 | potato soil | | | | | | | | | | |
| *P. protegens* | CHA0 | Tobacco root | 26.6 (24.3; 28.5) | a | 27.1 (1.3; 32) | a | | | | | | |
| *P. protegens* | K94.41 | Cucumber root | 20.3 | b | 26.5 (24.9; 27.7) | a | | | | | | |
| *P. protegens* | Pf-1 | Tobacco root | | | | | | | | | | |
| *P. protegens* | Pf5 | soil | | | | | | | | | | |
| *P. protegens* | PGNL1 | Tobacco root | | | | | | | | | | |
| *P. protegens* | PGNR2 | Tobacco root | | | | | | | | | | |
| *P. protegens* | PPRAR01 | Lithobius | | | | | | | | | | |
| *P. protegens* | PPRAR02 | Lithobius | | | | | | | | | | |
| *P. protegens* | PPRAR03 | Lepidoptera larvae | 27.5 (25.3; 29.5) | a | 25.6 (23; 27.2) | a | | | | | | |
| *P. protegens* | PPRAR04 | Agriotes | 26.7 (24.4; 28.5) | a | 25.7 (22.9; 27.5) | a | | | | | | |
| *P. protegens* | PPRAR05 | Staphylinidae | | | | | | | | | | |
| *P. protegens* | PPRAR06 | Agriotes | | | | | | | | | | |
| *P. protegens* | PPRAR07 | Lithobius | | | | | | | | | | |
| *P. protegens* | PPRAR08 | Geophilidae | | | | | | | | | | |
| *P. protegens* | PPRAR09 | Curculionidae | | | | | | | | | | |
| *P. protegens* | PPRAR10 | Agrypnus murinus | | | | | | | | | | |

| Species | Strain | isolation | Experiment 11 | | Experiment 12 | | Experiment 13 | | Experiment 14 | | Experiment 15 | | Experiment 16 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. chlororaphis* | PCL1391 | Tomato root | 24.7 | a | | | 24.6 (20; 27.5) | a | | | 26.9 (25.4; 29.9) | a | 19.8 (18.6; 20.8) | a |
| *P. chlororaphis* | 30.84 | Wheat seed | | | | | | | | | 47.5 (39.6; 54) | b | | |
| *P. chlororaphis* | PCLAR01 | Aphodiinae | | | | | | | | | | | | |
| *P. chlororaphis* | PCLAR02 | Aphodiinae | | | | | 46.4 (37.1; 56.8) | b | | | 56.5 (51.8; 60.8) | b | | |
| *P. chlororaphis* | PCLAR03 | Diptera | | | | | | | | | | | | |
| *P. chlororaphis* | PCLAR04 | Scarabaeidae pupa | | | | | | | | | | | | |
| *P. chlororaphis* | PCLAR05 | Scarabaeidae larva | | | | | | | | | | | 42.5 (40.1; 44.7) | b |
| *P. chlororaphis* | PCLIN01 | Lumbricidae | | | | | | | | | | | 29.9 (27.6; 32.1) | c |
| *P. chlororaphis* | PCLLF01 | Medicago | | | | | | | | | | | 22.2 (32.1; 36.5) | c |
| *P. chlororaphis* | PCLRT01 | grass root | 24.1 (22.7; 25.2) | a | | | | | | | | | | |
| *P. chlororaphis* | PCLRT02 | potato root | | | | | | | | | | | | |
| *P. chlororaphis* | PCLRT03 | potato root | | | | | | | | | | | | |
| *P. chlororaphis* | PCLRT04 | potato root | 31.7 (29.6; 34.5) | b | | | | | | | | | 29 (27.4; 31.1) | c |
| *P. chlororaphis* | PCLRT05 | grass root | | | | | | | | | | | 41 (37.5; 44.4) | b |
| *P. chlororaphis* | PCLSL01 | potato soil | 24.1 (22.7; 25.2) | a | | | | | | | | | | |
| *P. protegens* | CHA0 | Tobacco root | | | 32 (21.2; 40.2) | a | 27 (24.8; 29.5) | a | 26.4 (25.4; 27.3) | a | 30.6 (28.3; 37.6) | a | 23.8 (20; 27.9) | a |
| *P. protegens* | K94.41 | Cucumber root | | | | | | | 25.5 (24.3; 26.5) | a | | | | |
| *P. protegens* | Pf-1 | Tobacco root | | | 34.8 (32.1; 37.6) | a | 37.7 | b | | | | | 25.9 (18.2; 30.5) | a |
| *P. protegens* | Pf5 | soil | | | 36.8 (22.7; 54.2) | a | 28.1 (26.6; 30.7) | a | | | | | | |
| *P. protegens* | PGNL1 | Tobacco root | | | 34.1 (24.6; 46.7) | a | 26.3 (23.4; 28.9) | a | | | | | | |
| *P. protegens* | PGNR2 | Tobacco root | | | | | 28.7 (27.3; 32.3) | a | | | 33.2 (30.3; 35.8) | a | | |
| *P. protegens* | PPRAR01 | Lithobius | | | 25.9 (24.4; 28.5) | a | | | | | | | 22.7 (20.9; 24.6) | a |
| *P. protegens* | PPRAR02 | Lithobius | | | 26.6 (25.1; 29.7) | a | | | | | | | 23.4 (22.5; 24.2) | a |
| *P. protegens* | PPRAR03 | Lepidoptera larvae | | | | | | | 26.9 (26; 27.7) | a | | | | |
| *P. protegens* | PPRAR04 | Agriotes | | | | | | | 26.3 (25.3; 27.2) | a | | | | |
| *P. protegens* | PPRAR05 | Staphylinidae | | | 25 (23.7; 27) | a | | | | | | | 24.1 (23.1; 25.3) | a |
| *P. protegens* | PPRAR06 | Agriotes | | | 23.4 (22.3; 24.6) | a | | | | | | | | |
| *P. protegens* | PPRAR07 | Lithobius | | | 25.1 (24; 27) | a | | | | | | | | |
| *P. protegens* | PPRAR08 | Geophilidae | | | 24.8 (23.5; 26.8) | a | | | | | | | | |
| *P. protegens* | PPRAR09 | Curculionidae | | | 25.3 (24; 27.5) | a | | | | | | | | |
| *P. protegens* | PPRAR10 | Agrypnus murinus | | | 26.4 (25.1; 31) | a | | | | | | | | |

## Table S4. P-values of experiments depicted in Figure 2 and Supplementary Figure S2 and S3

| Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | CHA0 | 2.72E-13 | Experiment 1 | PPRAR01 | PCLRT01 | 3.28E-08 | Experiment 1 | PPRAR02 | CHA0 | 7.64E-05 | Experiment 3 |
| PCL1391 | CHA0 | 2.72E-13 | Experiment 1 | PPRAR05 | PCLRT01 | 5.90E-06 | Experiment 1 | PPRAR07 | CHA0 | 0.57020824 | Experiment 3 |
| PCLAR03 | CHA0 | 2.26E-12 | Experiment 1 | PPRAR06 | PCLRT01 | 7.20E-09 | Experiment 1 | PPRAR08 | CHA0 | 0.00160614 | Experiment 3 |
| PCLRT01 | CHA0 | 2.24E-10 | Experiment 1 | PPRAR10 | PCLRT01 | 1.29E-06 | Experiment 1 | PPRAR09 | CHA0 | 0.01314927 | Experiment 3 |
| PPRAR01 | CHA0 | 0.04783221 | Experiment 1 | PPRAR05 | PPRAR01 | 0.08230515 | Experiment 1 | PCL1391 | Control | 3.62E-10 | Experiment 3 |
| PPRAR05 | CHA0 | 0.0004144 | Experiment 1 | PPRAR06 | PPRAR01 | 0.11918613 | Experiment 1 | PCLAR04 | Control | 0.34857281 | Experiment 3 |
| PPRAR06 | CHA0 | 0.89723794 | Experiment 1 | PPRAR10 | PPRAR01 | 0.98171952 | Experiment 1 | PCLAR05 | Control | 3.44E-07 | Experiment 3 |
| PPRAR10 | CHA0 | 0.12670279 | Experiment 1 | PPRAR06 | PPRAR05 | 0.00471338 | Experiment 1 | PCLRT02 | Control | 1.86E-11 | Experiment 3 |
| PCL1391 | Control | 6.22E-07 | Experiment 1 | PPRAR10 | PPRAR05 | 0.23346753 | Experiment 1 | PCLRT04 | Control | 5.16E-12 | Experiment 3 |
| PCLAR03 | Control | 7.20E-09 | Experiment 1 | PPRAR10 | PPRAR06 | 0.23346753 | Experiment 1 | PPRAR02 | Control | 3.26E-14 | Experiment 3 |
| PCLRT01 | Control | 1.67E-07 | Experiment 1 | PCL1391 | Control | 5.55E-06 | Experiment 2 | PPRAR07 | Control | 6.63E-15 | Experiment 3 |
| PPRAR01 | Control | 5.65E-13 | Experiment 1 | PCLAR04 | Control | 0.05292768 | Experiment 2 | PPRAR08 | Control | 3.26E-14 | Experiment 3 |
| PPRAR05 | Control | 2.96E-12 | Experiment 1 | PCLAR05 | Control | 1.62E-05 | Experiment 2 | PPRAR09 | Control | 6.63E-15 | Experiment 3 |
| PPRAR06 | Control | 3.07E-13 | Experiment 1 | PCLRT02 | Control | 8.92E-08 | Experiment 2 | PCLAR04 | PCL1391 | 3.44E-07 | Experiment 3 |
| PPRAR10 | Control | 1.09E-12 | Experiment 1 | PCLAR04 | PCL1391 | 0.00211836 | Experiment 2 | PCLAR05 | PCL1391 | 0.20644608 | Experiment 3 |
| PCLAR03 | PCL1391 | 0.08569624 | Experiment 1 | PCLAR05 | PCL1391 | 0.80683158 | Experiment 2 | PCLRT02 | PCL1391 | 0.12633027 | Experiment 3 |
| PCLRT01 | PCL1391 | 0.67531493 | Experiment 1 | PCLRT02 | PCL1391 | 0.16606531 | Experiment 2 | PCLRT04 | PCL1391 | 0.11066766 | Experiment 3 |
| PPRAR01 | PCL1391 | 3.61E-11 | Experiment 1 | PCLAR05 | PCLAR04 | 0.00621746 | Experiment 2 | PPRAR02 | PCL1391 | 0.00018488 | Experiment 3 |
| PPRAR05 | PCL1391 | 7.20E-09 | Experiment 1 | PCLRT02 | PCLAR04 | 1.51E-05 | Experiment 2 | PPRAR07 | PCL1391 | 1.60E-08 | Experiment 3 |
| PPRAR06 | PCL1391 | 4.55E-11 | Experiment 1 | PCLRT02 | PCLAR05 | 0.05947418 | Experiment 2 | PPRAR08 | PCL1391 | 0.00011254 | Experiment 3 |
| PPRAR10 | PCL1391 | 1.29E-08 | Experiment 1 | Control | CHA0 | 6.63E-15 | Experiment 3 | PPRAR09 | PCL1391 | 2.35E-14 | Experiment 3 |
| PCLRT01 | PCLAR03 | 0.39355539 | Experiment 1 | PCL1391 | CHA0 | 5.30E-12 | Experiment 3 | PCLAR05 | PCLAR04 | 7.64E-05 | Experiment 3 |
| PPRAR01 | PCLAR03 | 3.80E-09 | Experiment 1 | PCLAR04 | CHA0 | 8.84E-14 | Experiment 3 | PCLRT02 | PCLAR04 | 1.47E-08 | Experiment 3 |
| PPRAR05 | PCLAR03 | 1.75E-06 | Experiment 1 | PCLAR05 | CHA0 | 9.51E-10 | Experiment 3 | PCLRT04 | PCLAR04 | 4.13E-09 | Experiment 3 |
| PPRAR06 | PCLAR03 | 2.66E-10 | Experiment 1 | PCLRT02 | CHA0 | 9.21E-09 | Experiment 3 | PPRAR02 | PCLAR04 | 1.86E-11 | Experiment 3 |
| PPRAR10 | PCLAR03 | 5.11E-07 | Experiment 1 | PCLRT04 | CHA0 | 3.85E-07 | Experiment 3 | PPRAR07 | PCLAR04 | 3.47E-13 | Experiment 3 |

| Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PPRAR08 | PCLAR04 | 1.52E-11 | Experiment 3 | PCL1391 | Control | 2.41E-06 | Experiment 4 | PPRAR04 | K94.41 | 0.00011619 | Experiment 6 |
| PPRAR09 | PCLAR04 | 2.31E-14 | Experiment 3 | PCLSL01 | Control | 8.86E-11 | Experiment 4 | PPRAR04 | PPRAR03 | 0.92873393 | Experiment 6 |
| PCLRT02 | PCLAR05 | 0.01843136 | Experiment 3 | PCLSL01 | PCL1391 | 0.00060796 | Experiment 4 | Control | CHA0 | 2.28E-15 | Experiment 7 |
| PCLRT04 | PCLAR05 | 0.01611466 | Experiment 3 | PCL1391 | Control | 4.71E-08 | Experiment 5 | K94.41 | CHA0 | 0.62132894 | Experiment 7 |
| PPRAR02 | PCLAR05 | 2.89E-05 | Experiment 3 | PCLAR03 | Control | 2.13E-07 | Experiment 5 | PPRAR03 | CHA0 | 0.62132894 | Experiment 7 |
| PPRAR07 | PCLAR05 | 1.12E-07 | Experiment 3 | PCLAR04 | Control | 0.18862024 | Experiment 5 | PPRAR04 | CHA0 | 0.7511234 | Experiment 7 |
| PPRAR08 | PCLAR05 | 2.28E-05 | Experiment 3 | PCLAR05 | Control | 3.89E-10 | Experiment 5 | K94.41 | Control | 9.24E-17 | Experiment 7 |
| PPRAR09 | PCLAR05 | 3.98E-11 | Experiment 3 | PCLRT02 | Control | 1.09E-10 | Experiment 5 | PPRAR03 | Control | 9.24E-17 | Experiment 7 |
| PCLRT04 | PCLRT02 | 0.91190047 | Experiment 3 | PCLAR03 | PCL1391 | 0.89641673 | Experiment 5 | PPRAR04 | Control | 9.24E-17 | Experiment 7 |
| PPRAR02 | PCLRT02 | 0.03193959 | Experiment 3 | PCLAR04 | PCL1391 | 6.19E-06 | Experiment 5 | PPRAR03 | K94.41 | 0.90694078 | Experiment 7 |
| PPRAR07 | PCLRT02 | 1.30E-05 | Experiment 3 | PCLAR05 | PCL1391 | 0.02813236 | Experiment 5 | PPRAR04 | K94.41 | 0.90694078 | Experiment 7 |
| PPRAR08 | PCLRT02 | 0.01941899 | Experiment 3 | PCLRT02 | PCL1391 | 0.01375576 | Experiment 5 | PPRAR04 | PPRAR03 | 0.90694078 | Experiment 7 |
| PPRAR09 | PCLRT02 | 6.14E-12 | Experiment 3 | PCLAR04 | PCLAR03 | 3.29E-05 | Experiment 5 | PCL1391 | Control | 8.16E-15 | Experiment 8 |
| PPRAR02 | PCLRT04 | 0.05302019 | Experiment 3 | PCLAR05 | PCLAR03 | 0.09366438 | Experiment 5 | PCLAR01 | Control | 2.76E-13 | Experiment 8 |
| PPRAR07 | PCLRT04 | 9.85E-05 | Experiment 3 | PCLRT02 | PCLAR03 | 0.06731575 | Experiment 5 | PCLAR03 | Control | 7.67E-16 | Experiment 8 |
| PPRAR08 | PCLRT04 | 0.03246491 | Experiment 3 | PCLAR05 | PCLAR04 | 2.80E-08 | Experiment 5 | PCLAR04 | Control | 1.99E-09 | Experiment 8 |
| PPRAR09 | PCLRT04 | 3.76E-09 | Experiment 3 | PCLRT02 | PCLAR04 | 2.80E-08 | Experiment 5 | PCLRT03 | Control | 1.28E-15 | Experiment 8 |
| PPRAR07 | PPRAR02 | 0.00528934 | Experiment 3 | PCLRT02 | PCLAR05 | 0.89641673 | Experiment 5 | PCLAR01 | PCL1391 | 0.0014284 | Experiment 8 |
| PPRAR08 | PPRAR02 | 0.70530202 | Experiment 3 | Control | CHA0 | 2.32E-15 | Experiment 6 | PCLAR03 | PCL1391 | 4.01E-06 | Experiment 8 |
| PPRAR09 | PPRAR02 | 5.67E-09 | Experiment 3 | K94.41 | CHA0 | 0.00011619 | Experiment 6 | PCLAR04 | PCL1391 | 0.01817993 | Experiment 8 |
| PPRAR08 | PPRAR07 | 0.03068512 | Experiment 3 | PPRAR03 | CHA0 | 0.92873393 | Experiment 6 | PCLRT03 | PCL1391 | 0.13497623 | Experiment 8 |
| PPRAR09 | PPRAR07 | 0.00923147 | Experiment 3 | PPRAR04 | CHA0 | 0.99404545 | Experiment 6 | PCLAR03 | PCLAR01 | 3.56E-10 | Experiment 8 |
| PPRAR09 | PPRAR08 | 7.86E-07 | Experiment 3 | K94.41 | Control | 2.96E-16 | Experiment 6 | PCLAR04 | PCLAR01 | 0.78890569 | Experiment 8 |
| Control | 30.84 | 5.25E-09 | Experiment 4 | PPRAR03 | Control | 1.05E-14 | Experiment 6 | PCLRT03 | PCLAR01 | 3.61E-06 | Experiment 8 |
| PCL1391 | 30.84 | 0.1982013 | Experiment 4 | PPRAR04 | Control | 2.32E-15 | Experiment 6 | PCLAR04 | PCLAR03 | 1.71E-07 | Experiment 8 |
| PCLSL01 | 30.84 | 0.00089468 | Experiment 4 | PPRAR03 | K94.41 | 2.24E-05 | Experiment 6 | PCLRT03 | PCLAR03 | 0.00020151 | Experiment 8 |

| Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PCLRT03 | PCLAR04 | 0.00056862 | Experiment 8 | PCLAR04 | PCLAR01 | 0.05580434 | Experiment 10 | PPRAR10 | CHA0 | 0.18995278 | Experiment 12 |
| PCL1391 | Control | 9.46E-16 | Experiment 9 | PCLRT03 | PCLAR01 | 0.00214708 | Experiment 10 | Pf-1 | Control | 1.81E-13 | Experiment 12 |
| PCLAR01 | Control | 2.09E-07 | Experiment 9 | PCLAR04 | PCLAR03 | 2.69E-08 | Experiment 10 | Pf5 | Control | 1.81E-13 | Experiment 12 |
| PCLAR03 | Control | 9.46E-16 | Experiment 9 | PCLRT03 | PCLAR03 | 5.76E-07 | Experiment 10 | PGNL1 | Control | 1.81E-13 | Experiment 12 |
| PCLAR04 | Control | 3.14E-10 | Experiment 9 | PCLRT03 | PCLAR04 | 0.21224444 | Experiment 10 | PPRAR01 | Control | 3.46E-13 | Experiment 12 |
| PCLRT03 | Control | 3.20E-14 | Experiment 9 | PCL1391 | Control | 3.76E-14 | Experiment 11 | PPRAR02 | Control | 7.75E-13 | Experiment 12 |
| PCLAR01 | PCL1391 | 3.68E-07 | Experiment 9 | PCLRT01 | Control | 3.76E-14 | Experiment 11 | PPRAR05 | Control | 2.65E-13 | Experiment 12 |
| PCLAR03 | PCL1391 | 0.79277584 | Experiment 9 | PCLRT04 | Control | 3.54E-13 | Experiment 11 | PPRAR06 | Control | 1.79E-13 | Experiment 12 |
| PCLAR04 | PCL1391 | 3.65E-05 | Experiment 9 | PCLSL01 | Control | 3.76E-14 | Experiment 11 | PPRAR07 | Control | 2.53E-13 | Experiment 12 |
| PCLRT03 | PCL1391 | 6.61E-06 | Experiment 9 | PCLRT01 | PCL1391 | 0.51362943 | Experiment 11 | PPRAR08 | Control | 2.53E-13 | Experiment 12 |
| PCLAR03 | PCLAR01 | 2.59E-07 | Experiment 9 | PCLRT04 | PCL1391 | 0.00032754 | Experiment 11 | PPRAR09 | Control | 3.46E-13 | Experiment 12 |
| PCLAR04 | PCLAR01 | 0.16934801 | Experiment 9 | PCLSL01 | PCL1391 | 0.5120834 | Experiment 11 | PPRAR10 | Control | 5.35E-13 | Experiment 12 |
| PCLRT03 | PCLAR01 | 0.00732757 | Experiment 9 | PCLRT04 | PCLRT01 | 1.91E-05 | Experiment 11 | Pf5 | Pf-1 | 0.78984102 | Experiment 12 |
| PCLAR04 | PCLAR03 | 1.09E-05 | Experiment 9 | PCLSL01 | PCLRT01 | 0.92070172 | Experiment 11 | PGNL1 | Pf-1 | 0.69876662 | Experiment 12 |
| PCLRT03 | PCLAR03 | 1.34E-06 | Experiment 9 | PCLSL01 | PCLRT04 | 1.70E-05 | Experiment 11 | PPRAR01 | Pf-1 | 0.00233543 | Experiment 12 |
| PCLRT03 | PCLAR04 | 0.33774676 | Experiment 9 | Control | CHA0 | 1.81E-13 | Experiment 12 | PPRAR02 | Pf-1 | 0.00778757 | Experiment 12 |
| PCL1391 | Control | 1.95E-16 | Experiment 10 | Pf-1 | CHA0 | 0.51989027 | Experiment 12 | PPRAR05 | Pf-1 | 0.00102105 | Experiment 12 |
| PCLAR01 | Control | 1.53E-11 | Experiment 10 | Pf5 | CHA0 | 0.28165805 | Experiment 12 | PPRAR06 | Pf-1 | 2.79E-05 | Experiment 12 |
| PCLAR03 | Control | 1.60E-16 | Experiment 10 | PGNL1 | CHA0 | 0.98791789 | Experiment 12 | PPRAR07 | Pf-1 | 0.00101363 | Experiment 12 |
| PCLAR04 | Control | 2.68E-14 | Experiment 10 | PPRAR01 | CHA0 | 0.0766304 | Experiment 12 | PPRAR08 | Pf-1 | 0.00075097 | Experiment 12 |
| PCLRT03 | Control | 2.64E-15 | Experiment 10 | PPRAR02 | CHA0 | 0.19034615 | Experiment 12 | PPRAR09 | Pf-1 | 0.00159853 | Experiment 12 |
| PCLAR01 | PCL1391 | 1.63E-09 | Experiment 10 | PPRAR05 | CHA0 | 0.03256748 | Experiment 12 | PPRAR10 | Pf-1 | 0.00738472 | Experiment 12 |
| PCLAR03 | PCL1391 | 0.42697676 | Experiment 10 | PPRAR06 | CHA0 | 0.00075097 | Experiment 12 | PGNL1 | Pf5 | 0.31574394 | Experiment 12 |
| PCLAR04 | PCL1391 | 4.64E-08 | Experiment 10 | PPRAR07 | CHA0 | 0.03071634 | Experiment 12 | PPRAR01 | Pf5 | 0.00031266 | Experiment 12 |
| PCLRT03 | PCL1391 | 1.27E-06 | Experiment 10 | PPRAR08 | CHA0 | 0.0228885 | Experiment 12 | PPRAR02 | Pf5 | 0.00102623 | Experiment 12 |
| PCLAR03 | PCLAR01 | 8.02E-10 | Experiment 10 | PPRAR09 | CHA0 | 0.04916071 | Experiment 12 | PPRAR05 | Pf5 | 0.00011732 | Experiment 12 |

| Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PPRAR06 | Pf5 | 8.71E-07 | Experiment 12 | PPRAR06 | PPRAR05 | 0.19786796 | Experiment 12 | Control | 30.84 | 9.20E-14 | Experiment 14 |
| PPRAR07 | Pf5 | 8.36E-05 | Experiment 12 | PPRAR07 | PPRAR05 | 0.97487529 | Experiment 12 | PCL1391 | 30.84 | 2.53E-09 | Experiment 14 |
| PPRAR08 | Pf5 | 8.09E-05 | Experiment 12 | PPRAR08 | PPRAR05 | 0.89726873 | Experiment 12 | PCLAR02 | 30.84 | 0.96549826 | Experiment 14 |
| PPRAR09 | Pf5 | 0.00019643 | Experiment 12 | PPRAR09 | PPRAR05 | 0.89245515 | Experiment 12 | Pf-1 | 30.84 | 0.00212692 | Experiment 14 |
| PPRAR10 | Pf5 | 0.00090692 | Experiment 12 | PPRAR10 | PPRAR05 | 0.35218278 | Experiment 12 | Pf5 | 30.84 | 7.82E-08 | Experiment 14 |
| PPRAR01 | PGNL1 | 0.05268323 | Experiment 12 | PPRAR07 | PPRAR06 | 0.13717873 | Experiment 12 | PGNL1 | 30.84 | 1.79E-08 | Experiment 14 |
| PPRAR02 | PGNL1 | 0.13702139 | Experiment 12 | PPRAR08 | PPRAR06 | 0.28165805 | Experiment 12 | PGNR2 | 30.84 | 1.98E-07 | Experiment 14 |
| PPRAR05 | PGNL1 | 0.0228885 | Experiment 12 | PPRAR09 | PPRAR06 | 0.13575499 | Experiment 12 | Control | CHA0 | 5.93E-14 | Experiment 14 |
| PPRAR06 | PGNL1 | 0.0005196 | Experiment 12 | PPRAR10 | PPRAR06 | 0.0129881 | Experiment 12 | PCL1391 | CHA0 | 0.73515117 | Experiment 14 |
| PPRAR07 | PGNL1 | 0.01978069 | Experiment 12 | PPRAR08 | PPRAR07 | 0.86828575 | Experiment 12 | PCLAR02 | CHA0 | 1.21E-06 | Experiment 14 |
| PPRAR08 | PGNL1 | 0.01729381 | Experiment 12 | PPRAR09 | PPRAR07 | 0.89753817 | Experiment 12 | Pf-1 | CHA0 | 0.00011155 | Experiment 14 |
| PPRAR09 | PGNL1 | 0.03300833 | Experiment 12 | PPRAR10 | PPRAR07 | 0.33230827 | Experiment 12 | Pf5 | CHA0 | 0.43212854 | Experiment 14 |
| PPRAR10 | PGNL1 | 0.13411662 | Experiment 12 | PPRAR09 | PPRAR08 | 0.78740955 | Experiment 12 | PGNL1 | CHA0 | 0.90753378 | Experiment 14 |
| PPRAR02 | PPRAR01 | 0.68739207 | Experiment 12 | PPRAR10 | PPRAR08 | 0.28165805 | Experiment 12 | PGNR2 | CHA0 | 0.18702571 | Experiment 14 |
| PPRAR05 | PPRAR01 | 0.70881693 | Experiment 12 | PPRAR10 | PPRAR09 | 0.465629 | Experiment 12 | PCL1391 | Control | 4.15E-14 | Experiment 14 |
| PPRAR06 | PPRAR01 | 0.06897388 | Experiment 12 | Control | CHA0 | 1.53E-16 | Experiment 13 | PCLAR02 | Control | 9.60E-13 | Experiment 14 |
| PPRAR07 | PPRAR01 | 0.70881693 | Experiment 12 | K94.41 | CHA0 | 0.76487707 | Experiment 13 | Pf-1 | Control | 9.94E-14 | Experiment 14 |
| PPRAR08 | PPRAR01 | 0.6174456 | Experiment 12 | PPRAR03 | CHA0 | 0.77853439 | Experiment 13 | Pf5 | Control | 1.39E-13 | Experiment 14 |
| PPRAR09 | PPRAR01 | 0.84262799 | Experiment 12 | PPRAR04 | CHA0 | 0.97695101 | Experiment 13 | PGNL1 | Control | 9.20E-14 | Experiment 14 |
| PPRAR10 | PPRAR01 | 0.66464731 | Experiment 12 | K94.41 | Control | 3.26E-16 | Experiment 13 | PGNR2 | Control | 2.24E-13 | Experiment 14 |
| PPRAR05 | PPRAR02 | 0.38829025 | Experiment 12 | PPRAR03 | Control | 5.76E-16 | Experiment 13 | PCLAR02 | PCL1391 | 1.27E-07 | Experiment 14 |
| PPRAR06 | PPRAR02 | 0.01978069 | Experiment 12 | PPRAR04 | Control | 1.53E-16 | Experiment 13 | Pf-1 | PCL1391 | 4.34E-06 | Experiment 14 |
| PPRAR07 | PPRAR02 | 0.36371096 | Experiment 12 | PPRAR03 | K94.41 | 0.64935867 | Experiment 13 | Pf5 | PCL1391 | 0.15398118 | Experiment 14 |
| PPRAR08 | PPRAR02 | 0.31574394 | Experiment 12 | PPRAR04 | K94.41 | 0.76487707 | Experiment 13 | PGNL1 | PCL1391 | 0.58787732 | Experiment 14 |
| PPRAR09 | PPRAR02 | 0.50335314 | Experiment 12 | PPRAR04 | PPRAR03 | 0.77853439 | Experiment 13 | PGNR2 | PCL1391 | 0.03768542 | Experiment 14 |
| PPRAR10 | PPRAR02 | 0.98791789 | Experiment 12 | CHA0 | 30.84 | 3.38E-08 | Experiment 14 | Pf-1 | PCLAR02 | 0.0195132 | Experiment 14 |

| Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment | Strain1 | Strain2 | p-value | Experiment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PPRAR06 | Pf5 | 8.71E-07 | Experiment 12 | PPRAR06 | PPRAR05 | 0.19786796 | Experiment 12 | Control | 30.84 | 9.20E-14 | Experiment 14 |
| PPRAR07 | Pf5 | 8.36E-05 | Experiment 12 | PPRAR07 | PPRAR05 | 0.97487529 | Experiment 12 | PCL1391 | 30.84 | 2.53E-09 | Experiment 14 |
| PPRAR08 | Pf5 | 8.09E-05 | Experiment 12 | PPRAR08 | PPRAR05 | 0.89726873 | Experiment 12 | PCLAR02 | 30.84 | 0.96549826 | Experiment 14 |
| PPRAR09 | Pf5 | 0.00019643 | Experiment 12 | PPRAR09 | PPRAR05 | 0.89245515 | Experiment 12 | Pf-1 | 30.84 | 0.00212692 | Experiment 14 |
| PPRAR10 | Pf5 | 0.00090692 | Experiment 12 | PPRAR10 | PPRAR05 | 0.35218278 | Experiment 12 | Pf5 | 30.84 | 7.82E-08 | Experiment 14 |
| PPRAR01 | PGNL1 | 0.05268323 | Experiment 12 | PPRAR07 | PPRAR06 | 0.13717873 | Experiment 12 | PGNL1 | 30.84 | 1.79E-08 | Experiment 14 |
| PPRAR02 | PGNL1 | 0.13702139 | Experiment 12 | PPRAR08 | PPRAR06 | 0.28165805 | Experiment 12 | PGNR2 | 30.84 | 1.98E-07 | Experiment 14 |
| PPRAR05 | PGNL1 | 0.0228885 | Experiment 12 | PPRAR09 | PPRAR06 | 0.13575499 | Experiment 12 | Control | CHA0 | 5.93E-14 | Experiment 14 |
| PPRAR06 | PGNL1 | 0.0005196 | Experiment 12 | PPRAR10 | PPRAR06 | 0.0129881 | Experiment 12 | PCL1391 | CHA0 | 0.73515117 | Experiment 14 |
| PPRAR07 | PGNL1 | 0.01978069 | Experiment 12 | PPRAR08 | PPRAR07 | 0.86828575 | Experiment 12 | PCLAR02 | CHA0 | 1.21E-06 | Experiment 14 |
| PPRAR08 | PGNL1 | 0.01729381 | Experiment 12 | PPRAR09 | PPRAR07 | 0.89753817 | Experiment 12 | Pf-1 | CHA0 | 0.00011155 | Experiment 14 |
| PPRAR09 | PGNL1 | 0.03300833 | Experiment 12 | PPRAR10 | PPRAR07 | 0.33230827 | Experiment 12 | Pf5 | CHA0 | 0.43212854 | Experiment 14 |
| PPRAR10 | PGNL1 | 0.13411662 | Experiment 12 | PPRAR09 | PPRAR08 | 0.78740955 | Experiment 12 | PGNL1 | CHA0 | 0.90753378 | Experiment 14 |
| PPRAR02 | PPRAR01 | 0.68739207 | Experiment 12 | PPRAR10 | PPRAR08 | 0.28165805 | Experiment 12 | PGNR2 | CHA0 | 0.18702571 | Experiment 14 |
| PPRAR05 | PPRAR01 | 0.70881693 | Experiment 12 | PPRAR10 | PPRAR09 | 0.465629 | Experiment 12 | PCL1391 | Control | 4.15E-14 | Experiment 14 |
| PPRAR06 | PPRAR01 | 0.06897388 | Experiment 12 | Control | CHA0 | 1.53E-16 | Experiment 13 | PCLAR02 | Control | 9.60E-13 | Experiment 14 |
| PPRAR07 | PPRAR01 | 0.70881693 | Experiment 12 | K94.41 | CHA0 | 0.76487707 | Experiment 13 | Pf-1 | Control | 9.94E-14 | Experiment 14 |
| PPRAR08 | PPRAR01 | 0.6174456 | Experiment 12 | PPRAR03 | CHA0 | 0.77853439 | Experiment 13 | Pf5 | Control | 1.39E-13 | Experiment 14 |
| PPRAR09 | PPRAR01 | 0.84262799 | Experiment 12 | PPRAR04 | CHA0 | 0.97695101 | Experiment 13 | PGNL1 | Control | 9.20E-14 | Experiment 14 |
| PPRAR10 | PPRAR01 | 0.66464731 | Experiment 12 | K94.41 | Control | 3.26E-16 | Experiment 13 | PGNR2 | Control | 2.24E-13 | Experiment 14 |
| PPRAR05 | PPRAR02 | 0.38829025 | Experiment 12 | PPRAR03 | Control | 5.76E-16 | Experiment 13 | PCLAR02 | PCL1391 | 1.27E-07 | Experiment 14 |
| PPRAR06 | PPRAR02 | 0.01978069 | Experiment 12 | PPRAR04 | Control | 1.53E-16 | Experiment 13 | Pf-1 | PCL1391 | 4.34E-06 | Experiment 14 |
| PPRAR07 | PPRAR02 | 0.36371096 | Experiment 12 | PPRAR03 | K94.41 | 0.64935867 | Experiment 13 | Pf5 | PCL1391 | 0.15398118 | Experiment 14 |
| PPRAR08 | PPRAR02 | 0.31574394 | Experiment 12 | PPRAR04 | K94.41 | 0.76487707 | Experiment 13 | PGNL1 | PCL1391 | 0.58787732 | Experiment 14 |
| PPRAR09 | PPRAR02 | 0.50335314 | Experiment 12 | PPRAR04 | PPRAR03 | 0.77853439 | Experiment 13 | PGNR2 | PCL1391 | 0.03768542 | Experiment 14 |
| PPRAR10 | PPRAR02 | 0.98791789 | Experiment 12 | CHA0 | 30.84 | 3.38E-08 | Experiment 14 | Pf-1 | PCLAR02 | 0.0195132 | Experiment 14 |

**Table S5. Number of mutations per gene and strain for the genes depicted in Figure 7 and Figure 8.** Synonymous mutations do not cause any aminoacid change. Non-synonymous mutations cause aminoacid change. The dN/dS ratio indicates negative (<1), neutral (=1) positive selection (>1)

| | Gene | Product | synonymous | non-synonymous | dN/dS | Total |
|---|---|---|---|---|---|---|
| PCLAR01 | C4K33_RS18300 | FitD | 101 | 41 | 0.40594059 | 142 |
| PCLAR01 | C4K33_RS10170 | ChiD | 15 | 3 | 0.2 | 18 |
| PCLAR01 | C4K33_RS15780 | PlcN | 8 | 1 | 0.125 | 9 |
| PCLAR01 | C4K33_RS19905 | TpsA2 | 183 | 88 | 0.48087432 | 271 |
| PCLAR01 | C4K33_RS21280 | TpsA4 | 0 | 0 | 0 | 0 |
| PCLAR01 | C4K33_RS08790 | TpsA1,3 | 28 | 40 | 1.42857143 | 68 |
| PCLAR03 | C4K33_RS18300 | FitD | 116 | 72 | 0.62068966 | 188 |
| PCLAR03 | C4K33_RS10170 | ChiD | 0 | 0 | 0 | 0 |
| PCLAR03 | C4K33_RS15780 | PlcN | 8 | 1 | 0.125 | 9 |
| PCLAR03 | C4K33_RS19905 | TpsA2 | 0 | 0 | 0 | 0 |
| PCLAR03 | C4K33_RS21280 | TpsA4 | 0 | 0 | 0 | 0 |
| PCLAR03 | C4K33_RS08790 | TpsA1,3 | 6 | 8 | 1.33333333 | 14 |
| PCLAR04 | C4K33_RS18300 | FitD | 101 | 41 | 0.40594059 | 142 |
| PCLAR04 | C4K33_RS10170 | ChiD | 15 | 3 | 0.2 | 18 |
| PCLAR04 | C4K33_RS15780 | PlcN | 8 | 1 | 0.125 | 9 |
| PCLAR04 | C4K33_RS19905 | TpsA2 | 182 | 89 | 0.48901099 | 271 |
| PCLAR04 | C4K33_RS21280 | TpsA4 | 0 | 0 | 0 | 0 |
| PCLAR04 | C4K33_RS08790 | TpsA1,3 | 27 | 37 | 1.37037037 | 64 |

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# Chapter 5

General Discussion

Final Remarks

This thesis focuses on *Pseudomonas*, a very versatile bacterial genus harbouring members able to colonize a wide range of environments and able to establish beneficial and pathogenic interactions [1]. Among the bacteria known for plant-beneficial interactions the *P. fluorescens* group is of special interest because it harbours excellent root colonizers, plant growth promoting rhizobacteria and biocontrol agents of soil pathogens as summarized in Figure 1 [2]. In 2008 it was discovered that, in addition to the plant-beneficial traits known so far, some species belonging to this group, i.e. *P. chlororaphis* and *P. protegens,* are able to invade and kill pest insects [3]. However, not all the tested insect species were equally susceptible [5]. At present it was not known if these bacteria were even able to invade non-susceptible insects and if they are common inhabitants of arthropods in nature. Although, several important factors contributing to insecticidal activity in *Pseudomonas* have been discovered in the last years, many important questions still remained unanswered. For example: when exactly during the pathogenicity process do the discovered factors play their role and how does colonizing an insect differ from colonizing a plant? These questions were addressed in chapters 2, 3 and 4 of this thesis. In the following sections, the major findings of each chapter are shortly summarized and their biological, ecological and agricultural relevance are discussed, as well as future perspectives. The major findings of this thesis are visualized in Figure 1 (dark-blue boxes).

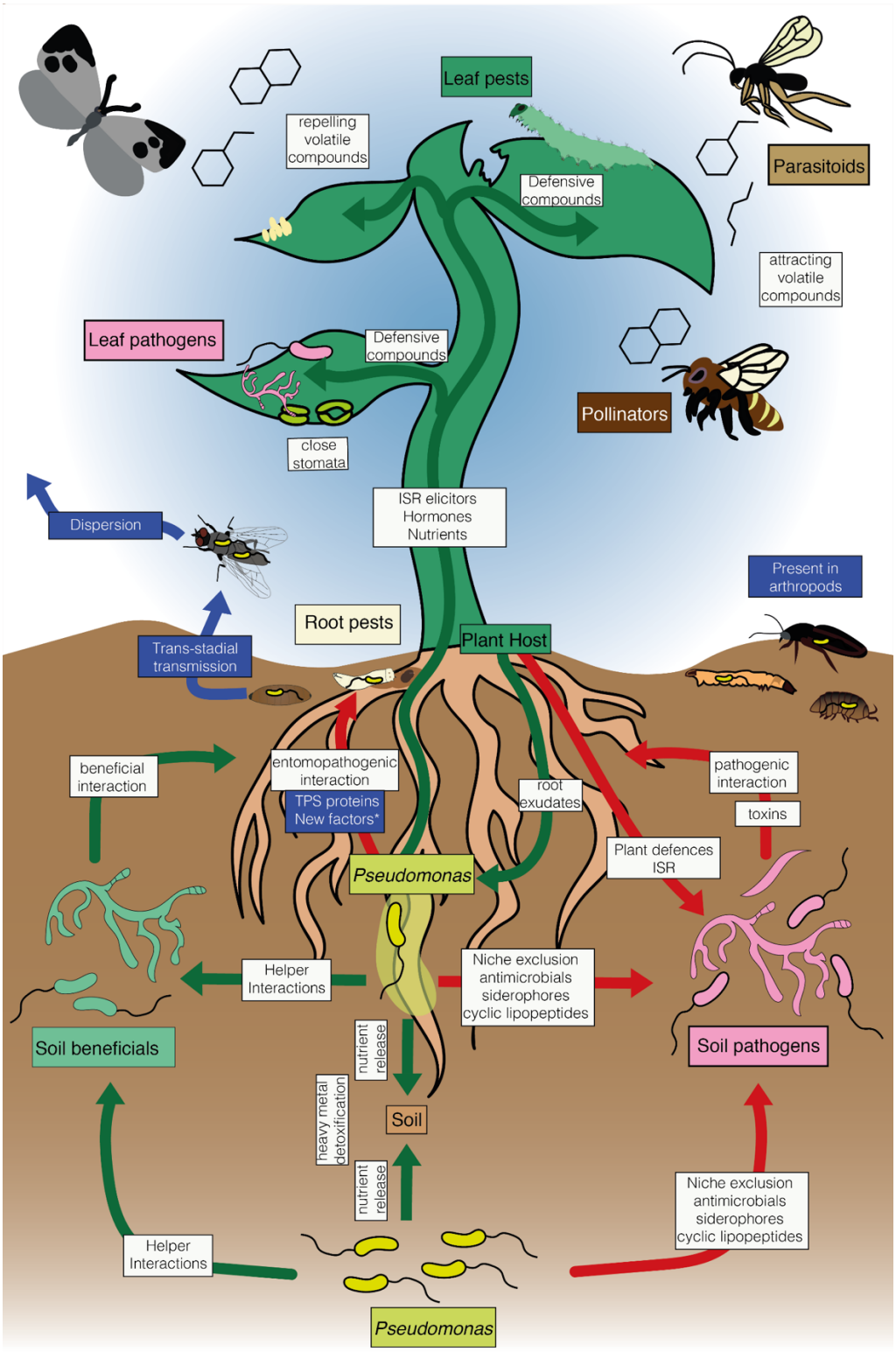


**Figure 1. Manifold interactions of beneficial pseudomonads with plant and insect hosts, including the discoveries made in this thesis.** *Pseudomonas* species can either live freely in the soil or associate to plant roots forming microcolonies, which use root exudates as nutrient source. They can promote plant growth directly by the production of growth hormones or indirectly by making soil

nutrients available, helping beneficial microbes to establish an interaction with the plant or by controlling pathogens and pest insects. They can control soil-borne pathogens through competitive colonization or production of antimicrobials and siderophores. *Pseudomonas* can also produce toxins that kill root pests. They can further induce systemic resistance in the plant resulting in an activation and acceleration of its fight against pathogens e.g. by producing effector molecules, closing stomata or accumulating toxic defensive compounds in damaged areas. *Pseudomonas* can also modify volatile production of the plants to repel pest insects or to attract pollinators and hyperparasites of pest insects. Green arrows indicate positive interactions; red arrows antagonistic interactions, and white boxes the *Pseudomonas* effect in a particular interaction. The new knowledge originated during this thesis is included in dark-blue boxes in the figure. *Pseudomonas* strains have been shown to be able to persist during the different insect life-stages from the larvae to the adult and further to be transmitted to a new plant host (see details in chapter 2). Insecticidal *Pseudomonas* strains were also found in arthropods captured in agricultural areas but, different isolates have variable insecticidal abilities in laboratory trials (see details in chapter 4). Finally, the infection window where known insecticidal factors act and new candidate proteins involved in insect pathogenesis were identified. Among those, the TPS proteins were demonstrated to play a role in the infection and it was hypothesized that they are important for the transition from gut to hemocoel and for killing insect immune cells (see details in chapter 3).

## 1. Insecticidal *Pseudomonas* are able to persist from larval to adult stage and can be transmitted to new plant hosts.

One open question regarding *Pseudomonas* insect interactions was whether the inability of certain pseudomonads to cause disease in certain insect species [4, 5] was due to the failure to persist in the insect. In chapter 2, *P. protegens* CHA0 was fed to *Delia radicum* (Diptera), *Plutella xylostella*, *Pieris brassicae* (Lepidoptera) and *Otiorhynchus sulcatus* (Coleoptera) larvae resulting in different effects for each insect species. The mortality of *D. radicum* and *O. sulcatus* larvae was not affected upon feeding on *P. protegens* CHA0 but in the Lepidoptera species, the final mortality of larvae and pupae

depended on bacterial doses and larval age. However, in all four species, bacteria were found in the adults, which implies that *P. protegens* CHA0 can persist through the life stages of all four insects even if they seemed healthy. Moreover, some adult animals emerged from *D. radicum*, *P. xylostella* and *P. brassicae* larvae fed with *P. protegens* CHA0 had anomalous morphologies which could possibly affect their fitness in nature (chapter 2). Anomalous animals are less likely to survive or to have offspring in nature, which probably will affect the number of larvae in the next generation that feed on the field. The *Pseudomonas* life-stage persistence and effects on insect development have been also shown for *Drosophila melanogaster* infected with Pf-5 [6, 7] and for the moth *Cnaphalocrocis medinalis* fed with rice leaves treated with a mix of *Pseudomonas* strains [8]. Additionally, our group found that *P. protegens* CHA0 can persist through different life-stages of *Melolontha melolontha* and *Agriotes obscurus* ([5], A. Spescha, unpublished data). Moreover, we showed that *P. protegens* CHA0 can be transmitted to a new host plant by healthy-looking *D. radicum* flies emerged from larvae treated with the bacteria ([9], chapter 2). By using insects as a vector, insecticidal *Pseudomonas* might spread to new environments, which might also affect insects invading these, e.g. freshly laid *D. radicum* eggs or other susceptible pest insects in agricultural fields.

Altogether, the findings of chapter 2 indicate that *Pseudomonas* bacteria can either be commensals or pathogens of insects, probably depending on the insect species, fitness and life stage. In addition, we showed that *Pseudomonas* can be dispersed in nature using insect vectors. The ecological relevance of the non-pathogenic interactions, is yet unclear and further addressed in chapter 4 and section 5.3.

*Pseudomonas* are able to invade and kill insects which is a very a challenging task. The insect gut is a very unfriendly environment with low pH, reactive oxygen and nitrogen species, antimicrobial peptides and the resident gut microflora. These stresses make the

persistence in the insect and the transition into the hemocoel very challenging. Moreover, holometabolous insects (those that undergo metamorphosis) remove all or almost all their gut microflora before pupation and acquire new resident microbes in the adult stage [10]. The fact that *Pseudomonas* strains can overcome these challenges and persist not only in the larval stage, but through the entire insect lifetime, shows that they must have a very special set of tools, which are further discussed in chapter 3 and section 5.2.

## 2. Insecticidal ability is a multifactorial trait in *Pseudomonas*

At first, the ability to kill insects was solely related to the Fit toxin but, shortly thereafter it was shown to be more complex, as mutants impaired for the production of this toxin remained insecticidal, although they were slower killers [3, 11, 12]. Since then, the insecticidal capabilities of fluorescent pseudomonads have been related to several factors, e.g. hydrogen cyanide [13], the cyclic lipopeptides orfamide [13, 14], sessilin [13] and Clp1391 [13], the type VI secretion system [15], a chitinase and a phospholipase [4], specific lipopolysaccharide O-antigens [16] and the toxins rhizoxin [6] and IPD072Aa [17]. However, even if the importance of all these factors was demonstrated, it was not clear at which time point of insect colonization and pathogenesis those factors were playing a role. Moreover, *Pseudomonas* are known for being plant growth promoters and biocontrol agents, therefore, we aimed to discover the different mechanisms required to infect an insect compared to colonize a plant root. To answer these questions, a comparative transcriptomic approach was chosen. The RNA of *P. protegens* CHA0 after being fed to *P. xylostella* larvae, injected to *Galleria mellonella* and inoculated on wheat roots was extracted and sequenced (chapter 3).

The RNA-seq analysis revealed that colonizing a plant-root and an insect require different mechanisms, the insect gut being a much more stressful environment for the

bacteria. Once the bacteria have survived the invasion of the gut and the transmigration into the hemocoel, they multiply rapidly as also shown in chapter 2 [9]. However, a big mystery of the *Pseudomonas* insect pathogenesis remained: how do they pass from the gut lumen into the hemocoel? The answer to this question is a major outcome of this thesis. Our RNA-seq data analysis enabled us to identify four complete two-partner secretion (TPS) protein systems in *P. protegens* CHA0 genome which were highly expressed in the different insect compartments. We named them *tpsBA1-4* due to the domain composition of these proteins which allow them to form a pore in the host-cell membrane and trigger cell death (chapter 3). Similar proteins have been related to macrophage pyropoptosis and epithelium disruption in *S. marcensces* and *P. aeruginosa* PA7 [18] and they seem to be important for insect pathogenesis as well. Mutants in *tpsA2* and *tpsA4* showed a delayed insecticidal activity upon feeding of *P. xylostella* and *tpsA4* mutant was also delayed when injected to *G. mellonella* larvae. These findings together with the other RNA-seq data allowed us to propose a pathogenesis model in *P. protegens* CHA0 (see chapter 3 for more details).

The outcome of the transcriptome comparisons underlines the fact that insect-*Pseudomonas* pathogenesis is a multifactorial trait involving many proteins and yet unknown regulatory factors. This evidences how versatile and adaptable this bacterial genus is and also makes insecticidal pseudomonads very interesting for pest control applications. Even if key factors are affected in the bacterial genome, they are still able to enter the hemocoel and kill the insect. This indicates that their biocontrol performance is stable and probably quite unsusceptible to the development of resistance in pest insects. This knowledge can be used to develop new plant protection products such as genetic modified organisms (introduction of interesting genes into crop plants or biocontrol agents), natural biopesticides based on specific insecticidal traits or bacterial inoculants.

Moreover, this knowledge allows us to identify soils, arthropods or other niches harbouring bacteria that possess these interesting features. Altogether, our research significantly adds to the development of new biological plant protection methods as an important contribution to integrated pest management and sustainable agriculture.

### 3. Insecticidal *Pseudomonas* inhabit arthropods in agricultural fields

The fact that *Pseudomonas* harbour insecticidal traits and show differences in insecticidal activity against different insect species, made us wonder whether these insecticidal bacteria are specific pathogens and also if they are naturally associated with insects. We showed in chapter 2 that *Pseudomonas* are able to establish non-pathogenic interactions with some insect pests in the laboratory, therefore, we wondered if insecticidal *Pseudomonas* were also associated with arthropods in agricultural fields. Previous studies have shown that *Pseudomonas* are usually present in the microbiota of different arthropod orders [19] but, to our best knowledge, there is no study so far, showing the occurrence of insecticidal *P. chlororaphis* or *P. protegens* in natural arthropods living in agricultural fields. Additionally, it is still unclear if *Pseudomonas* in arthropods are just transient bacteria acquired from the environment, or if they form close commensal interactions with the animal host. In our study, we collected arthropods, soil and roots from a potato field, a wheat field and a nearby undisturbed grassland in two consecutive years. We found that *P. protegens* and *P. chlororaphis* are indeed associated with the insect orders Diptera, Coleoptera and Lepidoptera as well as with myriapods. Interestingly, we did not find any *P. protegens* associated with plants or soil while *P. chlororaphis* were isolated from soil, roots and arthropods (chapter 4). In our earlier study we described two strains i.e. *P. chlororaphis* CD and *P. protegens* BRIP isolated from cyclops (Crustacea) [20]. Our unpublished data also show that bacteria belonging to the

*P. fluorescens* group occur in isopods (Crustacea), spiders (Arachnida), and some insects collected in ponds and creeks.

Besides their occurrence in arthropods, we further characterized the insecticidal and biocontrol abilities of the newly isolated *Pseudomonas* strains and compared them to some already characterized and published strains. We observed that *P. protegens* is a much more homogeneous group in terms of insect killing and colonization abilities while *P. chlororaphis* isolates are more variable, even though the new isolates are very phylogenetically close to each other. Interestingly, we found two *P. chlororaphis* Coleoptera isolates that were less insecticidal than the closely related reference strain *P. chlororaphis* PCL1391, a well-known insecticidal and plant growth promoting root-isolate. However, we have to keep in mind that the feeding assays were performed with laboratory reared insects and animals living in a natural environment could have different susceptibility. Additionally, experiments in Coleoptera species might also lead to a different outcome. In contrast to the insecticidal abilities, isolates from both species seemed to perform equally well in controlling the plant pathogen *P. ultimum*. To further investigate the differences in insecticidal activity between phylogenetically very closely related isolates, i.e. two less-pathogenic Coleoptera isolates, a highly pathogenic Diptera isolate and the highly pathogenic root isolate *P. chlororaphis* PCL1391; a single nucleotide polymorphism analysis of these strains was performed. The analysis revealed numerous mutations in coding sequences that lead to changes in the amino acids, which could affect the functionality of the protein. We specifically focused on the *fitD*, *chiD*, *plcN*, and *tpsA*-like genes, which were shown to have an impact on insecticidal activity in previous studies (see chapter 3 and section 5.2 for more details). Except for *tpsA4* and *plcN*, all these genes showed mutations that could affect the functionality of the protein. For *tpsA1*/3 we even found more non-synonymous than synonymous mutations, which

could be an indication that this gene is under selective pressure. All these modifications might be the reason why some *P. chlororaphis* strains are less insecticidal than the reference strain PCL1391 and might also be an indication for adaption towards a commensal interaction with the insect-host.

We concluded that the less insecticidal Coleoptera isolates might be commensals rather than pathogens of Coleoptera. The existence of a commensal *Pseudomonas*-Coleoptera interaction is supported by our previous results where *O. sulcatus* ([9], chapter 2), *Tenebrio mollitor*, *M. melolontha* and *A. obscurus* (our unpublished results) larvae showed none or very little susceptibility to *P. protegens* CHA0. Yet, the bacterium was shown to be able to persist until pupal and adult stage in three of these species. On the other hand, studies in which the dipteran *D. radicum* was fed with *P. protegens* and *P. chlororaphis* strains, *P. chlororaphis* seemed to have more impact on pupation rate and fly emergence ([9]; A. Spescha personal communication), which again points towards a certain insect-order specificity. The different behaviour of closely related *P. chlororaphis,* but not of closely related *P. protegens* strains observed in this study, can be an indication that the two species undergo different kinds of specializations, which would be very interesting for further studies. Our results indicate that, as *P. protegens* are a more homogeneous species in terms of insecticidal activity and insect colonization ability, and these findings might be extrapolated to the rest of the subgroup. In contrast, findings for *P. chlororaphis* have to be considered with more caution. Altogether, the results of chapter 2 and 4 data allow us to hypothesize a possible commensal relationship between *Pseudomonas* and certain insect species. There is still a lot to be discovered about the ecology of these amazing bacterial species with their multifactorial and agriculturally beneficial skills.

A commensal or opportunistic pathogenic relationship rises many evolutionary

questions concerning the *P. chlororaphis* and *P. protegens* groups. It is, for example, not clear if both species use exactly the same pathogenicity mechanisms. Furthermore, the discovered kinds of non-pathogenic relationships raise the question, whether bacteria colonizing the rhizosphere can actually modify plant volatiles in order to recruit insect species that would serve as vectors for the dispersal of the bacteria. All these questions remain to be further investigated in the future.

## 4. Future perspectives for research on *Pseudomonas* as biocontrol agents of insects and plant growth promoters

At the beginning of this thesis, several insecticidal factors where known but it was not clear when they were important in the insect pathogenesis and how colonizing a plant root differed from an insect-pathogenic interaction. The involvement of TPS-like proteins in insecticidal activity has been one of the biggest outcomes of this thesis. However, further microscopy studies that confirm the hypothetical activity of these proteins during insect pathogenesis, are needed. Furthermore, now it is evident that insecticidal activity depends on many different traits that need to be understood more in detail if we want to unravel the complexity of these interactions. However, we have to keep in mind that host-pathogen interactions are species-specific which calls for more studies with different strains of *Pseudomonas* and different insect orders since Lepidoptera so far are still the most studied. Our study provides very valuable information but it is only a moment in time during insect pathogenesis focused on the bacterial side. If we aim to fully understand the interactions, we need to study also the insect and plant host reactions. Unfortunately, most studies are currently focused on model organisms or very few bacterial strains. More molecular studies including more bacterial strains and insect species, e.g. transcriptomic studies investigating gene expression on the bacterium and the insect side are needed to

fully understand this pathogenic interaction.

It was also known that *Pseudomonas* are associated to arthropods but no-one had ever investigated if these bacterial strains belonged to the *P. chlororaphis* and *P. protegens* species or if they were insecticidal with a potential for biocontrol applications. We found that, indeed, these insecticidal species occur within several arthropod classes and, in some cases, we hypothesize that their interaction is rather commensal than pathogenic. Though, quantitative analyses of different insect orders and arthropod classes would be necessary to reveal if there are specific associations e.g. of specific types of pseudomonads to specific insect orders or if these bacteria can colonize any kind of susceptible animal. If the insect-*Pseudomonas* interaction turns out to be specific for certain species, it would be interesting to conduct experimental evolution experiments to observe if bacteria evolve to be more lethal or to be more commensals when repeatedly exposed to generations of susceptible insects. Since *Pseudomonas* are ubiquitous bacteria able to colonize and persist in very different niches [1], this might be rather a question of which mechanisms allow this bacterial genus to become insect pathogens or commensals and if these traits occur in other branches of the *Pseudomonas* tree. Finally, the dispersion potential has to be further investigated because beneficial insects could disperse *Pseudomonas* and maintain their numbers in the field which is still one of the biggest challenges in their application.

Additionally, more applied research is also needed because, no matter how complex a laboratory or greenhouse experiment is, it will never assemble all the environmental conditions the bacteria would be exposed to in nature. The current requirements for the development of new products for field application, are not only to increase the yield but also to not affect the crop quality and to have a minimal impact on non-target organisms and the biodiversity of the agroecosystem [21]. However, very few

studies focus on the effect of biocontrol agents on the plant nutrient and metabolite content and little is known about the effects on the soil microbiota under natural conditions. For instance, it was shown, that *Paenibacillus pasadenensis* R16 was able to control *Rhizoctonia solani* and *Pythium ultimum* infecting lettuce plants (*Lactuca sativa* L. var. *longifolia*) in greenhouse experiments. The bacteria did not affect the nutrient content of the plant host but it improved the texture of the leaves by reducing its mechanical resistance. Although the *P. pasadenensis* did not affect the composition of the surrounding soil microflora, the plant and rhizosphere microbiota were changed [22]. Such integrative field studies on potential impacts of bacterial inoculants on the resident microflora and on crop plant quality, are key requirements for the improvement of the biocontrol strategies.

In summary, for further progress in the development of *Pseudomonas*-based plant protection products, basic and applied science have to be combined to better understand pathogenesis, the ecological nature of insect relations, impact on crop quality and the interaction of these bacteria with their environment.

## 5. *Pseudomonas* potential as biocontrol agents in integrated pest management

Integrated pest management aims to use all the available tools to control pests in order to develop a more sustainable agricultural model. Chemical pesticides are less sustainable tools as they pollute water sources, affect wildlife and pose a risk to human health during application and as residues on crops [23, 24] . This does not mean that they should not be used at all, but they have to be applied in synergy with other more ecological methods such as biological control agents (BCAs) and their derived products. However, microbial BCAs are not a silver bullet for all the agricultural problems as an integrated solution gathers cultural practices, policy making, basic and applied science and marketing. BCAs are recognized as low risk agents. In general, BCAs are already present

in the agro-ecosystem, therefore, the risk of disturbing the soil microflora and crops is negligible [25]. So far, the most widely used bacterial BCA products are based on *Bacillus thuringiensis* but pests are becoming resistant to these products rendering the search for alternatives a necessity [26].

Fluorescent *Pseudomonas* strains are very promising BCA candidates as the variety of metabolites they produce have diverse action sites, which makes the development of resistances in pests less likely. They can be exploited in different ways e.g. as bacterial inoculants applied alone or in mixtures or as biopesticides or the metabolites they produce can be expressed in genetically modified plants. Commercial formulations of BCA have to resist UV radiation, environmental variations and storage for long periods of time. The biggest problem of *Pseudomonas* control agents is the storage of the product since they do not form spores like *B. thuringiensis*. On the market *Pseudomonas* formulations are only available as dehydrated powder and all of the products are meant to control soil and foliar pathogens and not pest insects [27, 28]. Other methods have been tried e.g. formulation as alginate beads, a promising method which allows, in addition, to combine pseudomonads with other entomopathogenic biocontrol agents such as entomopathogenic nematodes (our unpublished results). To combine several organisms with different mode of actions e.g. pseudomonads with entomopathogenic nematodes or entomopathogenic fungi could lead to a synergistic, or at least more stable biocontrol effect. The fungi and nematodes are, in contrast to the pseudomonads, able to infect the insect directly via the cuticle and could thereby carry the bacteria into the pest insect. Even if there are no commercial *Pseudomonas*-based insect control products available yet, preliminary studies combining nematodes and *P. chlororaphis* have shown an additive effect in insect killing which seems very promising for the development of future formulations (A. Spescha, unpublished results).

Additionally, several successful laboratory and field experiments have been performed to control insects using insecticidal *Pseudomonas* alone or in combination with other BCA's and different application techniques [29–31]. Even though these BCA combinations proved to be promising, the beneficial effect was not observed in every repetition of these field trials, which shows the current instability of such applications. Therefore, the next challenges on the way towards the development of successful *Pseudomonas*-based pest control products will be to find a stable formulation, to explore the possibility to improve their performance by using BCA-combinations, to prove that they do not pose any risks to environment, operators and consumers and finally to develop a product available for a reasonable price.

## 6. Final remarks

This thesis shows how *Pseudomonas* insect colonization differs from root colonization. In particular, it was determined at which time point during insect invasion already known factors and factors discovered in this thesis are expressed. As a special highlight, the importance of the two-partner secretion proteins for insect killing was discovered and their important role in transmigration from the gut lumen into the hemocoel proposed. These outcomes lead to the formulation of a pathogenesis model with several traits that need to be further investigated.

It was further discovered, that *P. protegens* and *P. chlororaphis* are common inhabitants of arthropods in agricultural fields and that they can persist through the life-stages of several holometabolous insects and even be transmitted to a new host-plant by insect vectors. The results of this work further indicate that insecticidal capabilities within the *P. chlororaphis/protegens* species are independent of phylogenetic position, but that the *P. chlororaphis* species is more heterogeneous regarding insecticidal activity than

previously thought. This raises the question if these bacteria are specialised insect pathogens, generalists or if the kind of interaction depends on the insect order or on specific genomic variations in the bacterial genome.

This thesis has answered many questions about the pathogenesis of insect infecting *Pseudomonas* and the ecology of *Pseudomonas*-insect interaction and has provided exciting new information important for the development of novel biocontrol tools. However, answers often bring more questions and insecticidal activity in *Pseudomonas* has emerged to be a puzzle with numerous molecular and ecological pieces; some of them we understand, for some of them we do not know where or how they fit and others still remain to be found. This leaves much room for future molecular and ecological research, which will unravel more secrets of these intriguing bacteria.

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# SCIENTIFIC CONTRIBUTIONS

## Published work

Martínez-Granero F, Redondo-Nieto M, **Vesga P**, Martín M, Rivilla R. AmrZ is a global transcriptional regulator implicated in iron uptake and environmental adaption in *P. fluorescens* F113. BMC Genomics. 2014 ;15:237.

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**Vesga P**., Flury P., Vacheron J., Keel C., Croll D., Maurhofer M. Transcriptome plasticity underlying plant root colonization and insect invasion by *Pseudomonas protegens*. ISME J, 1-17 (2020).

**Vesga P.**, Augustiny E., Keel C., Maurhofer M., Vacheron J. Phylogenetically closely related pseudomonads isolated from arthropods show differential insect killing abilities and genetic variations in insecticidal factors. In preparation.

## Oral presentations in conferences

**2019**

P. Vesga. Molecular determinants contributing to insect and plant associated lifestyles of beneficial *Pseudomonas*. Seminary cycle of the Institute of Integrative Biology/Plant Pathology, ETHZ, Zurich. 18 February 2019.

P. Vesga, C. Keel, D. Croll, M. Maurhofer. *Pseudomonas protegens* CHA0 transcriptome changes in response to root- and insect associated lifestyles. International Congress on Invertebrate Pathology and Microbial Control & 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests. July 28-August 1 2019, Valencia, Spain.

P. Vesga, C. Keel, D. Croll, M. Maurhofer. *Pseudomonas protegens* CHA0 transcriptome switches between root- and insect-associated lifestyles. Swiss Society for Microbiology Annual Meeting. September 3-4, 2019, Zurich, Switzerland.

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**2015**

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P. Vesga, Augustiny E., Flury P., Tinguely C., Keel C., Maurhofer M., Diversity in insect interactions within Pseudomonas protegens and P. chlororaphis strains isolated from roots and arthropods: adaptation to different hosts?

**2017**

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## Scientific communications to the common public

**2019**

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**2018**

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# CURRICULUM VITAE

Maria del Pilar Vesga Aguado
Born 31st July 1990
Citizen of Madrid, Spain

**2015 – 2020 Doctor of Science (PhD)**
ETH Zurich
Plant Pathology group, Institute of Integrative Biology
Doctoral Program: Plant Sciences
Title of the thesis: Plant-beneficial fluorescent pseudomonads with insecticidal activity: molecular traits and ecology of insect-associated lifestyles
Supervisors: Prof. Monika Maurhofer. ETH Zürich, and Dr. Christoph Keel, University of Lausanne.

**2018 – 2020 National webmaster of Pint of Science Switzerland**

**2014 – 2015 Predoctoral grant**
Universidad Autónoma de Madrid
Rhizosphere group, Biology department
Supervisors: Rafael Rivilla and Marta Martín

**2013 – 2014 Master (MSc) on Microbiology**
Universidad Autónoma de Madrid
Rhizosphere group, Biology department
Title master thesis: Estudio genómico de la variación de fase en *Pseudomonas fluorescens* F113 en la rizosfera
Supervisors: Miguel Redondo Nieto, Rafael Rivilla and Marta Martín

**2008 – 2013 Bachelor of Science (BSc)**
Universidad Autónoma de Madrid
Rhizosphere group, Biology department
16th best student of her promotion
Title bachelor project: ArmZ es un regulador transcripcional de genes implicados en la homeostasis de hierro en *Pseudomonas fluorescens* F113
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