DISS. ETH NO. 23940

GENOMICS, MOLECULAR MECHANISMS AND ECOLOGY OF INTERACTIONS OF PLANT-BENEFICIAL PSEUDOMONADS WITH PEST INSECTS

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

PASCALE CHRISTINA FLURY

M Sc in Plant Science, University of Basel
born on 07.03.1987
citizen of Basel (BS) and Balsthal (SO)

accepted on the recommendation of

Prof. Dr. Bruce McDonald
Dr. Monika Maurhofer
Prof. Dr. Philipp Engel
Dr. Christoph Keel

2016

TABLE OF CONTENTS

SUMMARY

This thesis focuses on the insecticidal activity of root-colonizing bacteria, phylogenetically belonging to the *Pseudomonas fluorescens* group. Strains of this group have been known for a long time for their plant beneficial effects, such as the suppression of soil-borne diseases, the induction of systemic resistance in the plant, and the promotion of plant growth. The discovery that certain strains have the additional ability to infect and kill insects, raised many questions about the molecular basis, the ecological relevance, and the agronomic potential of this trait. The main goal of this thesis was to gain a better understanding of these different aspects of insecticidal activity, with a focus on the infection process. We specifically aimed at identifying pathogenicity factors, assessing the role of insects as alternative hosts for dispersal, and exploring the insecticidal potential within the *P. fluorescens* group as reservoir for new biocontrol agents against insect pests.

In a first part, the phylogenetic distribution of plant-beneficial and insecticidal traits within the *P. fluorescens* group was investigated in experiments with twenty-six different strains. This revealed that strains with plant-beneficial traits are present throughout the entire *P. fluorescens* group, whereas strong insecticidal activity is restricted to a phylogenetically distinct sub-clade comprising the two species *Pseudomonas protegens* and *Pseudomonas chlororaphis*. It was hypothesized that strains with insecticidal activity may share specific traits that allow the infection of insects. In order to discover these traits, the genomes of twenty-four of the tested strains were sequenced and subjected to a comparative genomics analysis, which revealed over two hundred genes, common and unique to insecticidal strains. This selection of genes comprises the *fit* gene cluster, which is responsible for the production of Fit toxin, the only previously known insecticidal factor, but also several new, promising candidate toxicity factors. Four of them were subsequently deleted in the genome of model strain *P. protegens* CHA0 and mutants lacking either a specific chitinase or a phospholipase were found to exhibit attenuated virulence in oral infections of larvae of the diamondback moth, *Plutella xylostella*. In addition, mutants deficient for the production of various antimicrobial metabolites, which are important for the suppression of root-pathogenic fungi, were tested for insecticidal activity. This screening revealed that hydrogen cyanide along with different cyclic lipopeptides are essential for full virulence.

The aim of the second part of this thesis was to shed light on ecological aspects of insects as alternative hosts for plant-beneficial pseudomonads, especially on their potential to transmit the bacteria to a new host plant. The adult stage of root-feeding larvae generally emerges from the soil and searches for new host plants to deposit eggs, which could provide a mechanism for dispersal of rhizobacteria with otherwise very limited potential to spread over large distances. We tested *P. protegens* CHA0 for its ability to persist in insects upon ingestion by larvae, a prerequisite for dispersal. Indeed, the bacteria were transstadially transmitted to the pupal and the adult stage in three insect species of different orders. Furthermore, we could show that *P. protegens* CHA0 can be transmitted to the roots of a new plant host by cabbage root flies, *Delia radicum*, that had emerged from larvae feeding on CHA0-colonized roots, which gives first evidence for the possibility of insect-mediated dispersal of this bacterium by a root pest.

The results obtained in this thesis suggest the existence of different kinds of associations of plant-beneficial pseudomonads with insects, from commensal to pathogenic. In many cases, the bacteria are able to persist in insects and potentially use them as vectors, but in others they multiply to high numbers, thereby causing lethal infections and then use the insect cadaver as a source of nutrients. The dual activity of certain fluorescent pseudomonads against both root diseases and insect pests renders these bacteria interesting agents for biocontrol. However, further studies will be needed to comprehensively understand the mechanisms underlying insecticidal activity, to define the host range of different strains, and to assess the biocontrol potential of fluorescent pseudomonads against insects in the field. Moreover, future investigations may unravel specific adaptations that fluorescent pseudomonads have evolved to switch between plant-and insect-associated lifestyles, findings, which could also be of relevance for other bacteria which change between different hosts.

ZUSAMMENFASSUNG

Im Rahmen dieser Doktorarbeit wurde die insektizide Aktivität von wurzelkolonisierenden, phylogenetisch zur *Pseudomonas fluorescens* Gruppe gehörenden Bakterien untersucht. Vertreter dieser Gruppe von Bakterien waren für lange Zeit vor allem für ihre positiven Effekte auf Pflanzen bekannt: die Unterdrückung bodenbürtiger Pflanzenkrankheiten, die Induktion systemischer Resistenz in der Pflanze und die Förderung des Pflanzenwachstums. Mit der Entdeckung, dass gewisse Stämme der *P. fluorescens* Gruppe zusätzlich die Fähigkeit besitzen, Insekten zu infizieren und zu töten, kamen zahlreiche Fragen zu den molekularen Grundlagen, zur ökologischen Relevanz und zum agronomischen Potenzial dieser Eigenschaft auf. Das Hauptziel der vorliegenden Arbeit war es daher, neue Erkenntnisse zu den verschiedenen Aspekten insektizider Aktivität zu gewinnen. Der Schwerpunkt lag dabei auf der Untersuchung des Infektionsprozesses, im Speziellen der Suche nach Pathogenitätsfaktoren, der Erforschung der Rolle von Insekten als alternative Wirte für die Bakterienverbreitung und der Ermittlung des insektiziden Potenzials innerhalb der *P. fluorescens* Gruppe als Reservoir für neue Biokontrollagenzien gegen Schadinsekten.

In einem ersten Teil wurde anhand von sechsundzwanzig verschiedenen Stämmen experimentell die phylogenetische Verbreitung von pflanzenförderlichen und insektiziden Eigenschaften innerhalb der *P. fluorescens* Gruppe bestimmt. Dies ergab, dass Stämme mit pflanzenförderlichen Eigenschaften innerhalb der gesamten *P. fluorescens* Gruppe vorkommen, wohingegen sich starke insektizide Aktivität auf eine bestimmte phylogenetische Untergruppe beschränkt, welche die beiden Arten *Pseudomonas protegens* und *Pseudomonas chlororaphis* beinhaltet. In der Annahme, dass Stämme mit insektizider Aktivität bestimmte Eigenschaften teilen, welche die Infektion von Insekten ermöglichen, wurden die Genome von vierundzwanzig Stämmen sequenziert und eine vergleichende Genomanalyse durchgeführt. Diese identifizierte etwas über zweihundert Gene, welche den insektiziden Stämmen gemeinsam sind und dabei in keinem der Genome der nicht-insektiziden Stämme vorkommen. Diese Auswahl an Genen beinhaltet nebst dem *fit* Gen-Cluster, welches für die Produktion des Fit Toxins, dem einzigen vorgängig bekannten insektiziden Faktor, verantwortlich ist, mehrere neue, vielversprechende Kandidaten für Toxizitätsfaktoren. Deren vier wurden im Folgenden im Genom des Modellstamms *P. protegens* CHA0 deletiert und Mutanten, welchen entweder eine spezifische Chitinase oder Phospholipase fehlte, zeigten in oralen

Infektionen von Kohlschabenlarven, *Plutella xylostella*, eine verminderte Virulenz. Zusätzlich dazu wurden Mutanten untersucht, welche nicht in der Lage sind verschiedene antimikrobielle Metaboliten zu produzieren, welche wichtig sind für die Unterdrückung von wurzelpathogenen Pilzen. Dabei zeigte sich, dass Hydrogencyanid und verschiedene zyklische Lipopeptide für die Ausübung vollständiger Virulenz essenziell sind.

Im zweiten Teil dieser Arbeit war das Ziel, Aufschluss über ökologische Aspekte des Insekts als alternativen Wirt für pflanzenförderliche Pseudomonaden zu erlangen. Insbesondere ging es darum, zu untersuchen, ob die Möglichkeit besteht, dass die Bakterien durch Insekten auf neue Wirtspflanzen übertragen werden. Bei Arten mit wurzelfressendem Larvenstadium kommen die adulten Insekten in der Regel aus dem Boden heraus und suchen sich neue Wirtspflanzen zur Eiablage. Dabei könnten sie zur Verbreitung von Wurzelbakterien beitragen, welche ansonsten in ihren Möglichkeiten weite Distanzen zu überwinden stark eingeschränkt sind. Wir untersuchten, ob *P. protegens* CHA0 nach oraler Aufnahme durch das Larvenstadium im Insekt persistieren kann, eine Grundvoraussetzung für die Verbreitung des Bakteriums durch Insekten. In der Tat wurde *P. protegens* CHA0 in drei Insektenarten verschiedener Ordnungen transstadial an das Puppen- und das Adultstadium weitergegeben. Des Weiteren konnte gezeigt werden, dass *P. protegens* CHA0 mittels erwachsener Kohlfliegen, *Delia radicum*, welche als Larven CHA0-besiedelte Wurzeln gefressen hatten, von den Wurzeln einer Pflanze auf die Wurzeln einer anderen Pflanze gelangen kann. Diese Beobachtung zeigt erstmals auf, dass die Ausbreitung dieses Bakteriums mit Hilfe von Insekten möglich ist.

Die in dieser Arbeit generierten Resultate deuten darauf hin, dass verschiedene Arten von Assoziationen zwischen pflanzenförderlichen Pseudomonaden und Insekten existieren, von einer kommensalistischen bis hin zu einer pathogenen Lebensweise der Bakterien im Insekt. In vielen Fällen sind die Bakterien fähig in Insekten zu persistieren und nützen diese möglicherweise auch als Vektoren. In anderen Fällen hingegen vermehren sie sich stark, verursachen letale Infektionen und benützen anschliessend den Kadaver als Nahrungsquelle. Ihre duale Aktivität, gegen Wurzelkrankheiten und Schadinsekten, macht die hier untersuchten fluoreszierenden Pseudomonaden zu interessanten Agenzien für die Biokontrolle. Allerdings sind weitere Studien nötig, um die Mechanismen der insektiziden Aktivität vollständig zu verstehen, um das Wirtsspektrum verschiedener Stämme definieren zu können und, um das Biokontrollpotenzial gegen Insekten im Feld zu ermitteln. Zukünftige Untersuchungen

könnten zudem Aufschluss darüber geben, welche spezifischen Anpassungen diese Bakterien im Laufe der Evolution entwickelten, um den Wechsel zwischen einem Pflanzen- und einem Insekten-assoziierten Lebensstil zu bewältigen; Erkenntnisse, welche auch für viele andere Bakterien mit wechselnden Wirten von Relevanz sein könnten.

CHAPTER 1

General Introduction and Thesis Outline

1 GENERAL INTRODUCTION

1.1. Challenges in crop production - biological control as part of a solution

Since humankind started to cultivate land for food production around eleven thousand years ago, we were facing pests - diseases, animals and weeds - threatening our harvests. Nowadays, although agricultural practices changed tremendously and modern crop protection strategies have been developed, we are still losing substantial parts of crop yields each year. Oerke (2005) presents one of the rare estimations on crop loss according to which actual crop loss ranges between 15% and 60% depending on the region of the world, while the potential loss without crop protection would be globally even over 60%. The potential loss in eleven major cash and food crops due to pathogens and animal pests is estimated to be 16% and 18%, respectively (Oerke, 2005). As the world population is rising and thus food production has to be increased (Godfray *et al.*, 2010), improving plant protection will be a crucial asset in the next years. Moreover, circumstances like the ban of environmentally critical pesticides and the emergence of resistant pathogens and insect pests raise many challenges and demand the development of more sustainable and integral pest management practices.

One group of pests contributing considerably to yield losses comprises soil-borne diseases and insect herbivores attacking plant roots. These pests are often not only especially challenging to diagnose due to their cryptic way of life belowground, but are also difficult to combat. Although crop rotation can contribute to protection against some soil-borne pests, it is ineffective against others, for instance pathogens with a broad host range or long-lived spores (Agrios, 2005). Furthermore, pesticide application to soil is expensive and due to environmental concerns restricted in many countries. Alternatively, seeds can be treated with pesticides, an effective means against root herbivores, nematodes and many fungal diseases. However, increasing numbers of effective pesticides used for seed coatings are suspected to have adverse effect on the environment. A prominent example is the recent ban by the European Union of neonicotinoids, because of potential negative side effects on pollinators (Gross, 2013; van der Sluijs *et al.*, 2013). Neonicotinoids are globally very frequently applied chemical insecticides mostly used as seed coatings upon which they confer systemic resistance against root- and shoot-feeding insects. Because of their systemic nature they are also

present in reproductive organs and thus might become ingested by bees and other pollinators (Fairbrother *et al.*, 2014; Simon-Delso *et al.*, 2015).

In Switzerland a national action plan to reduce pesticide application is in preparation by the Swiss Federal Council and a strategy paper, which formulated the goal to reduce pesticide application by 40-50%, has been elaborated in a collaboration of many different organizations under the responsibility of Vision Landwirtschaft (Bosshard, 2016). Moreover, in organic agriculture, the use of chemical pesticides and genetically modified organisms has always been comprehensively prohibited. Hence, there is an apparent need for alternative crop protection strategies for conventional and organic farming in Switzerland and elsewhere.

Biological control represents a method of great, still developable potential that could account along with other measures, such as adaptations of agricultural practices and breeding for resistant cultivars, to a more environmentally friendly pest control in the future (Bale *et al.*, 2008). The concept of biological control is to confine the spread of a detrimental organism to an acceptable threshold by the use of living organisms as defined by Eilenberg *et al.* (2001). This can be achieved by introducing the biocontrol organism once (classical biocontrol) or repeatedly (inundative or augmentative biocontrol), if needed, or by modifying agricultural practices to become favorable to naturally occurring antagonists (conservation biocontrol) (Bale *et al.*, 2008).

1.2. The rhizosphere as a reservoir of biological control agents

The rhizosphere, the soil layer influenced by the root (Lugtenberg and Kamilova, 2009), is hosting diverse organisms, which are attracted by nutritious carbon compounds exuded by the plant. Vast numbers of bacteria, fungi, oomycetes, but also protozoans, nematodes, arthropods, earthworms and other organisms occupy this habitat. As little as one gram of rhizosphere soil can harbor billions of bacteria belonging to thousands of species; for instance, a metagenomic analysis by Mendes *et al.* (2011) detected over 33’000 bacterial and archaeal taxa in the rhizosphere of sugar beet. Unsurprisingly, this leads to many interspecific interactions not only amongst the rhizosphere inhabitants, but also directly with the plant and even indirectly via the plant with above-ground organisms. The idea of making use of these various interactions and of the species

richness of the rhizosphere in agricultural production systems is recently gaining increasing attention (Raaijmakers and Mazzola, 2016).

From an agronomical point of view, the focus lies on crop's growth performance and leads to the classification of rhizosphere inhabitants into 'beneficial' and 'harmful'. In this perspective, the goal of biological control is to protect crops from detrimental organisms by use of so-called 'beneficials'. However, from the perspective of the antagonized organisms these beneficials would be competitors if not even pathogens. Nevertheless, we will remain with the plant-centric definition of the terms in the following. Several groups of rhizosphere microorganisms have gained attention because of their capability to protect plants against pests and/or to promote plant growth. Here, an overview on biological control against root diseases and root-feeding insects is presented, with a focus on root-colonizing plant-beneficial pseudomonads.

1.3. Biological control of soil-borne diseases

Major soil-borne diseases are caused by fungal (e.g. *Fusarium*, *Rhizoctonia*, *Verticillium*, *Sclerotinia*, *Gaeumannomyces*, *Thielaviopsis* spp.), oomycete (*Phytophthora*, *Pythium* spp.), plasmodiophorid (*Spongospora*, *Plasmodiaphora* spp.) and bacterial (e.g. *Agrobacterium*, *Ralstonia*, *Streptomyces*, *Erwinia* spp.) pathogens and by plant parasitic nematodes (e.g. *Meloidogyne* spp.) (Agrios, 2005; Lugtenberg *et al.*, 2013a). The observation that in some soils certain diseases do not occur, although the causative pathogen is present, so called disease suppressive soils, led to the discovery that infections by root pathogens can be reduced by beneficial microbes resident in these soils (Weller *et al.*, 2002; Haas and Défago, 2005; Raaijmakers and Mazzola, 2016). Microbial-induced disease suppression is mediated by different mechanisms including competition, antagonism, induced systemic resistance (ISR) and mycoparasitism (Agrios, 2005).

Well-established plant-beneficial microbes include bacteria of the Proteobacteria (*Pseudomonas*, *Burkholderia*, *Rhizobium* spp.) and Firmicutes (*Bacillus* spp.) as well as fungi (*Trichoderma*, *Gliocladium* spp. and arbuscular mycorrhizal fungi)(Raaijmakers *et al.*, 2009; Lugtenberg *et al.*, 2013b). To date several products containing these organisms are available on the market for disease control and as plant fortifiers (Stockwell and Stack, 2007; Berg, 2009), but their number is still rather low (Parnell *et*

al., 2016). Reasons for this are amongst others the risk of inconsistent effects of living microbes being influenced by many environmental factors and the long registration processes, which can become disproportionally expensive compared to the expectable profit (Ehlers, 2011).

1.4. Plant-beneficial effects of fluorescent pseudomonads

Many strains of the genus *Pseudomonas* have reported activity against soil-borne diseases and most of them phylogenetically belong to the *Pseudomonas fluorescens* and the *Pseudomonas putida* group, but also the *Pseudomonas aeruginosa* group harbors strains with biocontrol activity (Figure 1). However, these strains are not of interest for agronomical applications, because of their inherent risks as close relatives to human pathogens. Plant-beneficial effects of *P. fluorescens* group bacteria have been extensively studied over the last decades and important findings are summarized in several comprehensive reviews (Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Weller, 2007; Lugtenberg and Kamilova, 2009; Raaijmakers *et al.*, 2009; Berendsen *et al.*, 2012; Kupferschmied *et al.*, 2013).

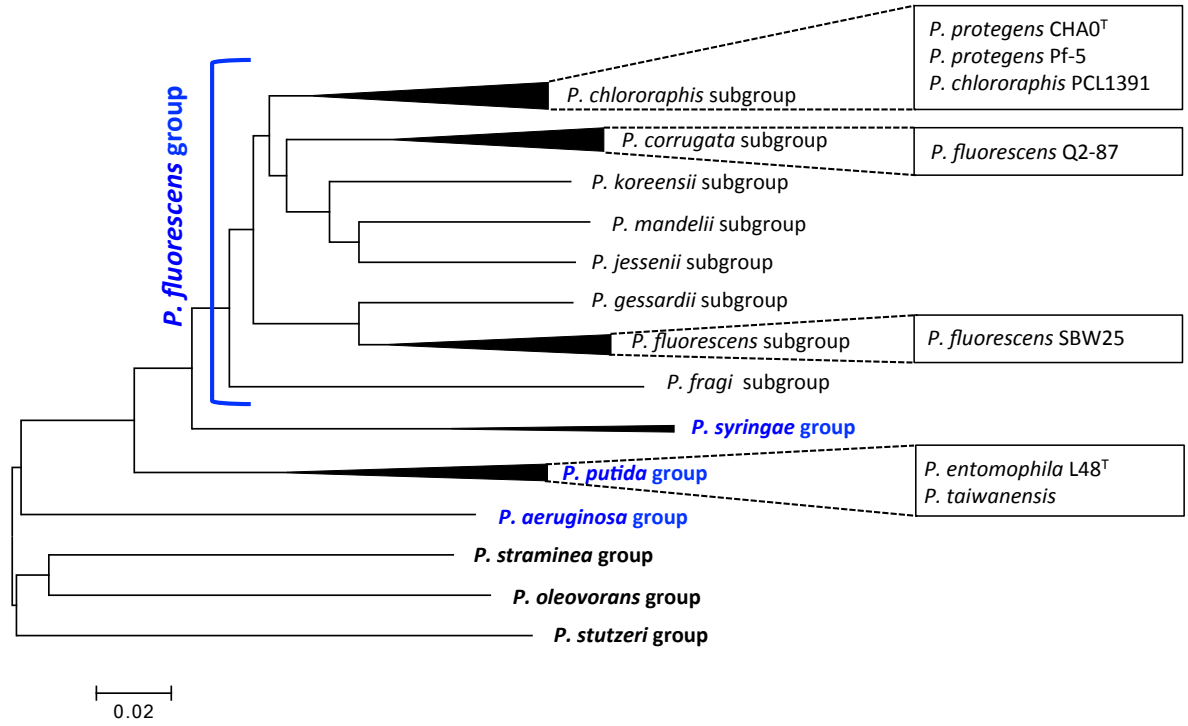


Figure 1 Phylogeny of the genus *Pseudomonas* incorporating important groups (blue) and strains discussed in this chapter. The core genome tree was generated using EDGAR 2.1 (Blom *et al.*, 2009; Blom *et al.*, 2016).

Within the *P. fluorescens* group prominent biocontrol strains are found in the *P. chlororaphis*, the *P. corrugata,* and the *P. fluorescens* subgroups (Figure 1). Fluorescent pseudomonads with biocontrol activity are commonly generalists that are able to efficiently colonize roots of various host plants, reaching upon inoculation population sizes around 10^7 to 10^8 colony forming units (cfu) per g of roots. In the rhizosphere, they directly compete with pathogenic fungi for nutrients and space (Mercado-Blanco, 2015). Probably the most important mechanism in suppression of root diseases by this group of pseudomonads is the production of antimicrobial compounds. Most biocontrol strains produce a strain specific cocktail of secondary metabolites, such as 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, phenazines, hydrogen cyanide and different cyclic lipopeptides (Raaijmakers *et al.*, 2002; Haas and Keel, 2003; Haas and Défago, 2005; Gross and Loper, 2009; Loper *et al.*, 2012). Beside the relatively local action of antimicrobial compounds, several strains also induce defense responses in the host plant, which render the entire plant more resistant to pathogen attack (Bakker *et al.*, 2007; De Vleesschauwer and Höfte, 2009). This phenomenon, called induced systemic resistance (ISR), was in recent studies found to act also against insects feeding on above-ground plant parts (Saravanakumar *et al.*, 2007; Van Oosten *et al.*, 2008; Pineda *et al.*, 2010; van de Mortel *et al.*, 2012). In addition to their antimicrobial effects, many fluorescent pseudomonads exhibit the ability to promote plant growth by the mobilization of nutrients, especially phosphate, and the production of phytohormones (Lugtenberg and Kamilova, 2009).

One strain that proved to be a multi-talent exhibiting all the above-mentioned features is *Pseudomonas protegens* CHA0 (formerly *P. fluorescens* CHA0)(Stutz *et al.*, 1986; Keel *et al.*, 1989; Voisard *et al.*, 1989; Keel *et al.*, 1990; Keel *et al.*, 1992; Laville *et al.*, 1992; Maurhofer *et al.*, 1992; Maurhofer *et al.*, 1994; Iavicoli *et al.*, 2003; Meyer *et al.*, 2011; Ramette *et al.*, 2011), the main bacterial model organism investigated in this thesis (Figure 1). To illustrate, how effective the biocontrol effect exhibited by *P. protegens* CHA0 can be, pictures of cucumber plants infected with *Pythium ultimum*, in presence or absence of CHA0 are depicted in Figure 2. While in the treatment without the biocontrol bacteria no seedlings are able to emerge, almost all plants in the CHA0 treatment survive. For the disease suppressive activity, the production of antimicrobial compounds is crucial. Accordingly, a mutant defective for the global regulatory system GacS/GacA, which controls the production of antimicrobial compounds (Laville *et al.*,

1992; Haas and Keel, 2003), is unable to protect cucumber seeds from the pathogen (Figure 2).

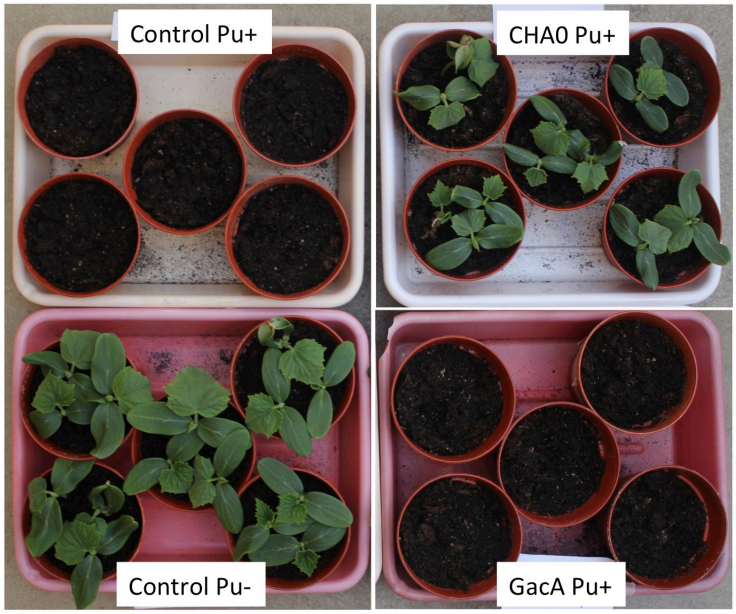


Figure 2 Suppression of cucumber damping-off disease caused by *Pythium ultimum* (Pu) by a plant-beneficial fluorescent pseudomonad. Pots were inoculated with (Pu+) or without (Pu-) the pathogen. When the biocontrol bacterium *Pseudomonas protegens* CHA0 (CHA0) is added, seedling survival is higher than in the treatment without added bacteria (Control Pu+). The major mechanism by which CHA0 suppresses the disease is by antibiosis. A GacA deficient mutant of CHA0 (GacA), unable to produce antimicrobial compounds, does not exhibit the biocontrol effect.

1.5. Biological control of insect pests

Biological control displays also an effective alternative to synthetic pesticides in the control of pests caused by herbivorous arthropods which represent another major threat to crop production. Moreover, biocontrol can also contribute to control of insects with medical or veterinary relevance, for example against vectors of major human diseases such as malaria (Kamareddine, 2012). Biocontrol agents generally have an advantage over synthetic insecticides in terms of safety to humans and the environment, but their production is more cost-intensive, it mostly takes them longer to kill the host and they are susceptible to negative environmental influences (Shapiro-Ilan *et al.*, 2012).

Different groups of insect pathogenic organisms are currently applied for biocontrol. Certain entomopathogenic fungi, e.g. *Metarhizium* spp. and *Beauveria* spp.

are well-established biocontrol agents. They are often highly virulent towards a broad range of insect hosts, which they can penetrate upon contact (Shapiro-Ilan *et al.*, 2012). An advantage of many fungal biocontrol agents is the feasibility of mass production and storage. However, they are acting rather slowly and, as a consequence, extensive crop damage may occur before the pest population becomes controlled.

Another successfully applied group of biocontrol agents are viruses, mainly baculoviruses. They generally exhibit a stringent host specificity, which makes them especially safe in terms of side-effects against beneficial insects (Harrison and Hoover, 2012). On the other hand, a narrow host range can be a disadvantage in case of threat by different insect pests at the same time. To date viral based products are mainly successfully applied against major lepidopteran pests such as *Helicoverpa* spp. and *Spodoptera* spp. A disadvantage of viruses is, that they can only be produced in vivo which leads to high production costs (Lacey *et al.*, 2015). In contrast, the nowadays most widely applied bacterial and nematode biocontrol agents can be produced at large scale (Shapiro-Ilan *et al.*, 2012). Furthermore, these two groups of biocontrol agents generally cause rapid insect death upon infection.

Entomopathogenic nematodes of the genus *Heterorhabditis* and *Steinernema* harbor bacterial mutualist of the genus *Photorhabdus* and *Xenorhabdus*, respectively. Together they kill the insect host rendering it into a food source and breeding ground (Lewis and Clarke, 2012). The nematode partner penetrates into the insect body cavity and releases the insecticidal bacteria into the insect blood system, where these multiply and finally cause insect death by a combination of toxins and septicemia. *Photorhabdus* and *Xenorhabdus* produce several insect toxins, amongst the most important ones are toxin complexes (Tc's) and 'makes caterpillars floppy' (Mcf) (ffrench-Constant *et al.*, 2007). Tc's are genetically complex and variants occur also in many other bacterial species in many of which their biological function is still unclear (Hurst *et al.*, 2004; Hares *et al.*, 2008; Blackburn *et al.*, 2011; Loper *et al.*, 2012). The Mcf toxin can induce apoptosis in insect cells and causes loss of body turgor (floppy phenotype) (Daborn, 2002; Waterfield *et al.*, 2003).

The most prominently applied biocontrol bacterium is *Bacillus thuringiensis*, accounting for over 50% of market share of all microbial control agents against arthropod pests (Lacey *et al.*, 2015). This gram-positive spore-producing bacterium exhibits advantages of chemical and microbial pesticides - fast killing, cheap production,

long shelf-life and at the same time high levels of safety to the environment (Lacey *et al.*, 2015). Different subspecies of *B. thuringiensis* (Bt) are specific to distinct groups of insect host species. Beside the use as bacterial sprays, Bt derived crystal proteins (Cry) and vegetative insecticidal proteins (Vip) (Palma *et al.*, 2014; Chakroun *et al.*, 2016) were expressed in major crop species and these transgenic plants with insect resistance became commercially highly successful in many parts of the world. However, resistance against Bt-derived toxins is increasingly observed in several major pest species (Tabashnik *et al.*, 2013; Crickmore, 2016). Therefore, extensive research to find new bacterial toxins with insecticidal activity is ongoing and Gram-negative bacteria, especially the genus *Pseudomonas* (see Chapters 1.7 and 1.8), have entered the focus of interest as biocontrol agents or reservoir of insect toxins.

1.6. The insect defense against intruding pathogens

Insects have evolved different features protecting their tissues from intruding pathogens. In a first line of defense the cuticle encompassing the entire body builds a physical barrier. In contrast to entomopathogenic fungi, which are able to invade the insect hemocoel directly by breaching the cuticle (Roy *et al.*, 2006), bacteria rely on alternative invasion strategies. Entomopathogenic bacteria like *Photorhabdus* and *Xenorhabdus* gain access to the hemolymph by their association to nematodes, free living insect pathogenic bacteria, however, typically infect their host upon ingestion. Infection through respiratory systems, which is known from mammals, has not been documented in insects (Vallet-Gely *et al.*, 2008).

The physiology of the insect digestive tract can differ between species and stadium within the life-cycle and is adapted to the mode of nutrition. Nevertheless, it can generally be divided into foregut, midgut and hindgut (Chapman, 1998). While foregut and hindgut are lined with the cuticle, the midgut epithelial cells are often protected by a peritrophic matrix (PM), a semipermeable structure consisting of chitin, proteins, such peritrophin, and proteoglycans (Figure 3) (Lehane, 1997; Chapman, 1998). The PM protects the epithelial cells against large particles and against pathogens, but it lets digestive enzymes and nutrients pass. In addition, the existence of a PM causes a compartmentalisation of the midgut lumen into an endoperitrophic space and an ectoperitrophic space (Figure 3).

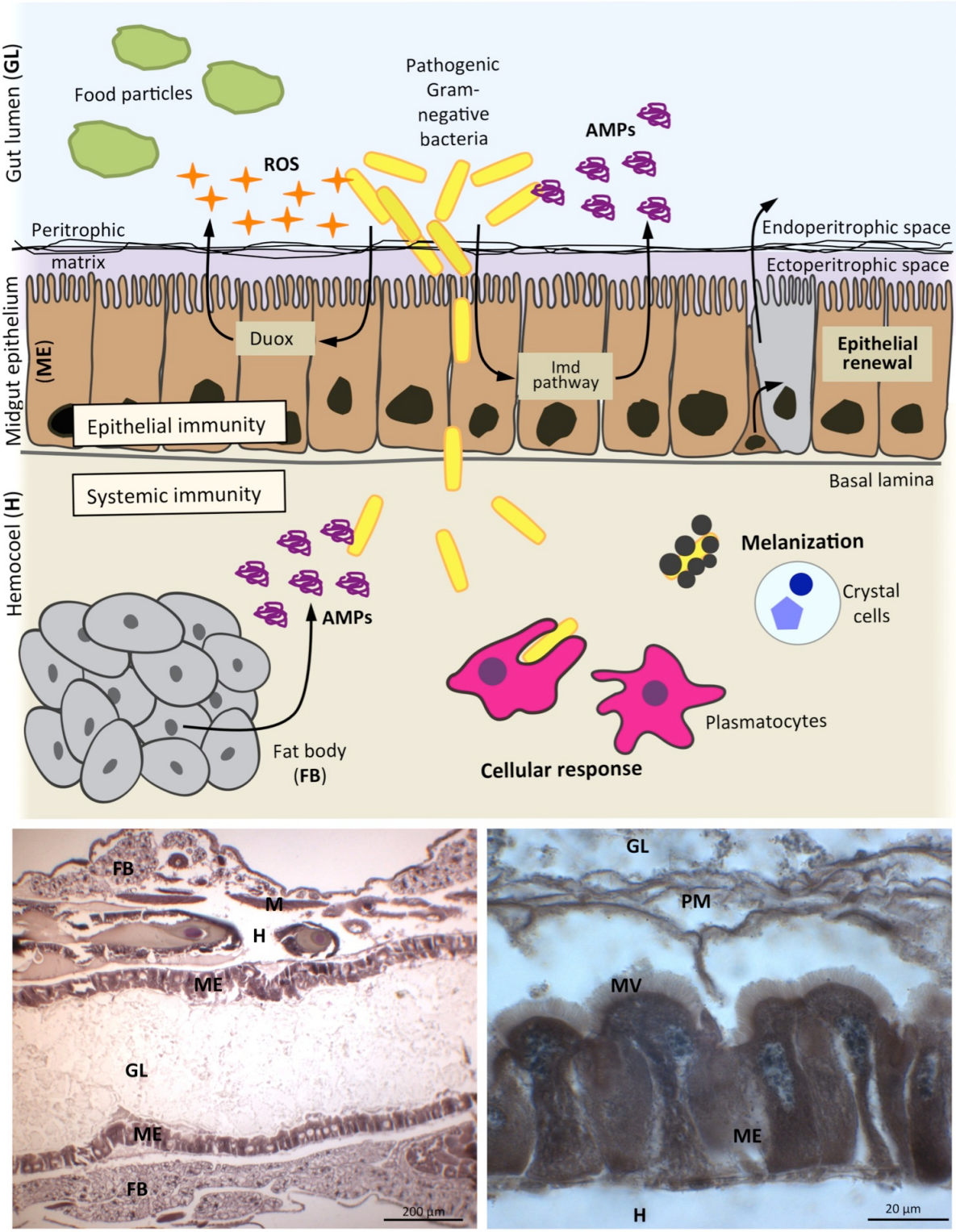


Figure 3 Insect physiology and immunity. Top: Insect immune response towards intruding Gram-negative bacteria. Gram-negative bacteria in the midgut are restrained from the living insect cells by hostile conditions in the gut lumen, the peritorphic matrix and the epithelial immune response (production of reactive oxygen species (ROS) and antimicrobial peptides (AMPs)). Bacteria that are able to enter the hemocoel face a systemic immunity (AMP production by the fat body, phagocytosis and melanization). Damaged midgut epithelial cells become replaced by epithelial renewal from instestinal stem cells. For details see text. **Bottom:** Histological section of a healthy *Plutella xylostella* larva. FB, fat body; GL, gut lumen; H, hemocoel; Imd, Immune deficiency pathway; M, muscle; ME, midgut epithelium; MV, microvilli brush border; PM, peritorphic matrix.

In the latter a countercurrent flow of liquids may enhance nutrient uptake by prolongation of the digestive period (Lehane, 1997). The PM can be seen as a tolerance mechanism, since it reduces the impact of bacteria on the midgut epithelium without reducing their numbers (Engel and Moran, 2013). In contrast, extreme pH, e.g. very alkaline regions in the midguts of many lepidopteran, coleopteran and dipteran species (Appel and Martin, 1990; Harrison, 2001) and the production of lytic enzymes can restrict bacterial growth (Buchon *et al.*, 2013). Despite these different protection mechanisms, the midgut represents in most cases the site where bacterial pathogens attack to access the insect body cavity, unless entrance is possible through wounds in the integument.

The insect immune system has been extensively studied in the model organism *Drosophila melanogaster*. Recognition of Gram-negative bacteria in the midgut induces the so called epithelial immunity, the generation of reactive oxygen species (ROS) by the DUOX system and the production of antimicrobial peptides (AMPs; Figure 3) (Vallet-Gely *et al.*, 2008). The regulation of AMP production in the gut occurs via the Immune deficiency (Imd) signaling pathway upon recognition of bacterial peptidoglycan by receptors of the peptidoglycan recognition protein family (Buchon *et al.*, 2013). Microbes that are able to invade the hemocoel are facing a systemic immune response (Figure 3). Fat body cells release various molecules in response to infection amongst which are several classes of AMPs with a broad range of activities against bacteria and/or fungi (Lemaitre and Hoffmann, 2007). Moreover, a cellular response is triggered, represented by specialized phagocytizing blood cells, mainly plasmatocytes, which eliminate foreign cells. Alongside, a melanization response, mediated by crystal cells protects the insect against pathogens. The fragile crystal cells, which store prophenoloxidases in a crystallized form, easily disrupt and release their content into the hemolymph where the activated phenoloxidases are involved in the production of melanin. Several intermediates of melanin production are cytotoxic and therefore thought to contribute to killing of bacteria (Meister, 2004).

During the infection process pathogens cause damage to the tissues, which is repaired by different mechanisms. In a coagulation reaction clots are formed around the injured site preventing further invasion of the pathogen. Bacterial toxins, but also the insect's immune reaction, especially the production of ROS, can damage the midgut epithelium causing delamination of enterocytes. By an epithelial renewal program,

these cells are then replaced by newly synthesized cells emerging from intestinal stem cells (Figure 3)(Buchon *et al.*, 2013).

To overcome the insect immune system, entomopathogenic bacteria have evolved different strategies. They may avoid detection by the immune system, be resistant to the immune response e.g. against AMPs or suppress the immune response. Moreover, they produce toxins, such as Cry toxins, that directly harm the insect tissues (Vallet-Gely *et al.*, 2008).

1.7. Insecticidal activity in the genus *Pseudomonas*

Beside plant-beneficial strains, the genus *Pseudomonas* (Figure 1) harbors also pathogenic bacteria. Best known are probably the opportunistic human pathogen *Pseudomonas aeruginosa* and the plant pathogen *Pseudomonas syringae*. Both species also have reported potential to infect and kill insects as recently reviewed by Kupferschmied *et al.* (2013). The insect pathogenicity of *P. aeruginosa* mainly gained attention, because it allows to study virulence mechanisms of clinical isolates in *D. melanogaster*, a suitable model, whose innate immunity relies on similar mechanisms to the ones of mammalians (Vallet-Gely *et al.*, 2008). However, insect infections by *P. aeruginosa* might also be of relevance in natural insect populations, since several *P. aeruginosa* strains have been isolated from sick insects and were, following the Koch's postulates, identified as the causative agent for the disease (Osborn *et al.*, 2002; Haloi *et al.*, 2016). Several factors have been identified to contribute to insecticidal activity of *P. aeruginosa*, amongst them are Type 3-secreted effectors, quorum sensing, hydrogen cyanide, pyoverdine, superoxide dismutase, exotoxin A, and the Gac regulatory system (Chugani *et al.*, 2001; Fauvarque *et al.*, 2002; Miyata *et al.*, 2003; Avet-Rochex *et al.*, 2005; Chieda *et al.*, 2005; Iiyama *et al.*, 2007; Borlee *et al.*, 2008; Broderick *et al.*, 2008; Okuda *et al.*, 2010; Chieda *et al.*, 2011; Limmer *et al.*, 2011). In general, there is a high variability in virulence towards insects amongst different *P. aeruginosa* strains (Hilker *et al.*, 2015). Similar strain specific differences seem to exist also in the *P. syringae* group. While most *P. syringae* strains are probably not able to cause lethal infections in insects, *P. syringae* pv. *syringae* B728 was reported to orally infect and kill the pea aphid *Acyrthosiphon pisum*. However, the molecular basis of this activity is still elusive (Stavrinides *et al.*, 2009).

Over the last ten years, a third *Pseudomonas* group, the *P. putida* group (Figure 1), gained attention in terms of insecticidal activity. Strains phylogenetically clustering in the *P. putida* group are frequently found as soil and water inhabitants and many of them are of interest, because of their plant-beneficial traits or their bioremediation capacities. To date, two representatives of the *P. putida* group have been found to exhibit insecticidal activity (Vodovar *et al.*, 2005; Liu *et al.*, 2010). The supernatant of *Pseudomonas taiwanensis* cultures was found to cause significant mortality when ingested by *D. melanogaster* larvae and this activity was found to be at least partly attributed to a TccC-like toxin produced by this strain (Liu *et al.*, 2010). The more intensively studied insect pathogenic pseudomonad of the *P. putida* group, however, is *Pseudomonas entomophila* L48T. This strain was isolated from *D. melanogaster* in the framework of a screen for bacterial pathogens (Vodovar *et al.*, 2005). Virulence of *P. entomophila* L48^T is multifactorial and several general regulators - GacA, PrtR, and AlgR - have major effects on virulence (Liehl *et al.*, 2006; Vodovar *et al.*, 2006; Opota *et al.*, 2011). Moreover, the metallo-protease AprA, the pore-forming toxin monalysin and the *Pseudomonas* virulence factor (Pvf), possibly a signaling molecule, are reported to contribute to insecticidal activity of *P. entomophila* (Liehl *et al.*, 2006; Vallet-Gely *et al.*, 2010b; Opota *et al.*, 2011; Dieppois *et al.*, 2015). Upon ingestion, *P. entomophila* persists in the gut and damages the midgut epithelium by the production of pore-forming toxins, which can cross the PM (Vodovar *et al.*, 2005; Dieppois *et al.*, 2015). In addition, the midgut cells are harmed by the production of ROS, which are produced by the insect as an immune response to *P. entomophila*. The cellular damage excessively activates stress pathways, which leads to translational inhibition in midgut epithelial cells (Chakrabarti *et al.*, 2012). This means, although genes encoding for AMPs are expressed, AMPs are no longer produced. Besides preventing immune pathways, translational arrest also blocks epithelial renewal, a mechanism crucial for repair of damaged tissue (Buchon *et al.*, 2013), and this finally leads to the collapse of the midgut gut epithelium and insect mortality (Dieppois *et al.*, 2015). Thus, *P. entomophila* takes advantage of an overreaction of the insect immune system to infect *D. melanogaster*.

Although *P. entomophila* possesses specific adaptations to infect *D. melanogaster*, it has been hypothesized that it is rather an opportunistic pathogen (Dieppois *et al.*, 2015). It causes lethal oral infections of *D. melanogaster* only when applied at very high doses (OD_{600} of 50 or 100) and therefore it is unclear to which extent it is pathogenic

beyond laboratory conditions. However, since *D. melanogaster* feeds on rotting fruits, on which bacterial numbers can reach very high numbers, *P. entomophila* infections might still be of relevance in natural populations (Dieppois *et al.*, 2015).

1.8. Insecticidal activity in the *Pseudomonas fluorescens* group

Finally, the *Pseudomonas* group discovered most recently to harbor insecticidal strains is the *P. fluorescens* group (Figure 1), which includes many strains with biocontrol activity against plant diseases (see Chapter 1.4.). When the first entire genomic DNA sequences became available (Paulsen *et al.*, 2005), a gene closely related to that encoding the insect toxin Mcf1 of *Photorhabdus luminescens* was detected in the genome of *P. protegens* Pf-5 and CHA0 (ffrench-Constant *et al.*, 2007; Péchy-Tarr *et al.*, 2008). The encoded toxin was named Fit, for *P. fluorescens* insecticidal toxin (Péchy-Tarr *et al.*, 2008). The Fit toxin encoding gene *fitD* is part of an entire gene cluster consisting of eight genes (*fitABCDEFGH*). *fitABC* and E are predicted to encode a type I secretion system, while *fitFGH* were found to regulate toxin production (Péchy-Tarr *et al.*, 2008; Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014). Indeed both strains, Pf-5 and CHA0, were demonstrated to be highly toxic when injected into the hemocoel of the greater wax moth *Galleria mellonella* and the tobacco horn worm *Manduca sexta* (Péchy-Tarr *et al.*, 2008).

This finding opened up a completely new view on the lifestyle of these bacteria. Formerly well-studied as root-colonizing plant-beneficial bacteria, they suddenly had also to be considered as insect pathogens, which raised a plethora of questions about infection mechanisms, evolution of insecticidal activity, ecological relevance and last but not least about potential applications of this trait in insect control. Several studies have been conducted to tackle these questions mainly focusing on the role of the Fit toxin. In the above-mentioned injection experiments with *P. protegens* CHA0 and Pf-5 several lines of evidence suggested an important role for Fit in insecticidal activity. Larvae, injected with *P. protegens*, developed a strong melanization response and a floppy phenotype as described for Mcf (Péchy-Tarr *et al.*, 2008). Mutants with deletions in the Fit toxin gene *fitD* exhibited significantly decreased mortality. Furthermore, heterologous expression of *fitD* from an inducible promoter in *Escherichia coli* sufficed to render these naturally avirulent bacteria highly toxic causing lethal infections when

injected into *G. mellonella* larvae (Péchy-Tarr *et al.*, 2008).

P. protegens CHA0, Pf-5 and *Pseudomonas chlororaphis* PCL1391, another *fit* carrying strain of the *P. fluorescens* group (Figure 1), were shown to exhibit also potent oral insecticidal activity (Figure 4)(Olcott *et al.*, 2010; Ruffner *et al.*, 2013). *P. protegens* CHA0 and *P. chlororaphis* PCL1391 fed to three leaf-feeding insect pests of agronomic importance, i.e. the African cotton leafworm, *Spodoptera littoralis*, the diamondback moth, *Plutella xylostella,* and the tobacco budworm, *Heliothis virescens,* caused significant mortality already within a few days, whereas *Pseudomonas fluorescens* Q2-87, a related strain naturally deficient for the *fit* genes, did not affect larval survival (Ruffner *et al.*, 2013). *P. protegens* Pf-5 ingested by larvae of *D. melanogaster* caused significant mortality and developmental delay, in particular delayed pupation of larvae, in a dose dependent manner (Olcott *et al.*, 2010). Moreover, flies emerging from Pf-5 infected larvae showed morphological defects, most frequently eyes with abnormally small size and/or anterior and posterior nicks (Olcott *et al.*, 2010).

Figure 4 Lethal infections of the diamondback moth, *Plutella xylostella*, caused by *Pseudomonas protegens* CHA0 after oral uptake. **Left:** healthy control larva. **Middle:** Larva that succumbed to infection with *P. protegens* CHA0. **Right:** Dead *P. xylostella* larvae are highly colonized by CHA0. Extract of a moribund larva infected with a CHA0 derivative constitutively expressing GFP, examined by fluorescence microscopy.

Oral insecticidal activity is much rarer than injectable activity and is thought to require specific adaptations to be able to persist the insect immune response and breach the gut barrier. Similar to results obtained by direct injection of bacteria into the hemocoel, a *fit* deletion mutant of CHA0 and PCL1391 showed reduced oral insecticidal activity towards *S. littoralis* larvae. In contrast to injection experiments, oral application of *fit* expressing *E. coli* did not result in significant mortality of *S. littoralis* larvae indicating that additional factors are required for oral toxicity (Ruffner *et al.*, 2013).

Moreover, though being an important component of virulence against certain insect species, Fit seems to be dispensable in other *Pseudomonas*-insect interactions. Whereas Fit contributes to oral insecticidal activity of Pf-5 against *M. sexta* larvae (Rangel *et al.*, 2016) a *fit* deficient mutant did not significantly differ from the wild type in infections of *D. melanogaster* larvae (Loper *et al.*, 2016). Nevertheless, Fit seems to be a specific adaptation to life in an insect host, which is supported by findings on the evolution and regulation of *fit* genes (Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014; Ruffner *et al.*, 2015).

1.9. Evolution and regulation of Fit toxins

Recently, Fit toxin was discovered to be specifically produced in insects, but not on plant roots (Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014). Two studies unraveling *fit* regulation using mutants and quantitative single cell microscopy revealed that *fitD* is specifically expressed in certain insect species, i.e. *S. littoralis* and *Tenebrio molitor*, but not in other insects as demonstrated for *Acyrthosiphon pisum* and neither on roots of cucumber and wheat nor on roots of tomato plants with and without infection by the pathogen *Fusarium oxysporum* (Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014). Moreover, *fitD* was expressed in cells growing in an insect hemolymph-mimicking medium and expression could be suppressed by the addition of root extracts to the medium (Kupferschmied *et al.*, 2014). This indicates that *P. protegens* CHA0 is able to regulate *fit* expression in a host/environmental specific manner. The current model suggests, that the sensor histidine kinase FitF is the primary sensor of a yet to be discovered signal (Kupferschmied *et al.*, 2014).

In the insect host, a conformational change and subsequent phosphotransfer reactions of FitF inactivates the repressor FitH, which causes derepression of the LysR type transcriptional activator FitG finally leading to expression of *fitABCDE* (Kupferschmied *et al.*, 2014). FitF evolved by a domain-shuffling event by which a common sensor domain had been recruited from a regulatory protein involved in control of nutrient uptake. This evolutionary step represents an adaptation to an insect-pathogenic lifestyle as it enabled specific toxin expression in insects, but not on plant roots (Kupferschmied *et al.*, 2014). The authors hypothesized that the signal perceived by FitF may be a plant molecule that directly or indirectly inhibits FitF which could

explain the absence of toxin production on roots and in insect media supplemented with root extracts (Kupferschmied *et al.*, 2014).

Evolution of the *fit* genes was further investigated in a study by Ruffner *et al.* (2015). They found the majority of genes of the *fit* cluster to be most similar to genes outside the *Pseudomonas* genus, which, together with an atypical GC composition in the *fit* region, indicates that *fit* genes, at least partly, evolved via horizontal gene transfer. This event probably was followed by additional functional integration of vertically transmitted genes, leading to a unique *Pseudomonas*-specific insect toxin cluster (Ruffner *et al.*, 2015). The *fit* genes are only present in genomes of strains of the *Pseudomonas chlororaphis* subgroup (Figure 1), but not in any other *Pseudomonas* species (Ruffner *et al.*, 2015). Ruffner and colleagues further tested insecticidal activity of thirty strains of the *P. fluorescens* group by injection into *G. mellonella* larvae. Whereas all *fit* carrying strains exhibited potent insecticidal activity, *fit* negative strains did not cause significant mortality. Presence of the *fit* genes was thus suggested to be a potential molecular marker, which is predictive for high insecticidal activity even though, depending on the bacteria-host interaction, other virulence factors might be more important (Ruffner *et al.*, 2015).

While non-fit carrying strains tested by Ruffner *et al.* (2015) were completely avirulent, other work identified several strains of the *P. fluorescens* subgroup, e.g., *Pseudomonas fluorescens* SBW25 (Figure 1), to exhibit insecticidal activity towards *D. melanogaster* and/or *M. sexta* (Olcott *et al.*, 2010; Rangel *et al.*, 2016). However, the insect toxicity of these strains was rather inconsistent and much weaker compared to *P. protegens* Pf-5 and was, in addition, only demonstrated by experiments with very few larvae and repetitions (Rangel *et al.*, 2016). Hence, the extent to which insecticidal activity occurs in strains outside the *P. chlororaphis* subgroup remains to be investigated more thoroughly.

1.10. Additional toxicity factors related to insecticidal activity of the *P. chlororaphis* subgroup

While Fit seems to be dispensable in oral infections of certain insects, a functional GacS/GacA regulatory system was found to be crucial for full virulence in all tested insect species (Olcott *et al.*, 2010; Ruffner *et al.*, 2013). Accordingly, a *gacA* deficient mutant of *P. protegens* CHA0, which still produces the Fit toxin (Kupferschmied, 2015), caused decreased mortality compared to the wild type when fed to *S. littoralis* larvae (Ruffner *et al.*, 2013). Moreover, a double mutant, lacking *fitD* and *gacA*, was more strongly reduced in virulence than the respective single mutants (Ruffner *et al.*, 2013). These findings indicate the existence of additional, Gac-regulated, pathogenicity factors. When this thesis was started, none of them had been identified and to discover them was a major formulated goal of this research project (see Chapters 2 and 3).

Recently, investigations with *P. protegens* Pf-5 revealed additional toxicity factors. Mutants deficient in the production of a chitinase and the cyclic lipopeptide orfamide A showed reduced virulence in oral infections of *D. melanogaster* (Loper *et al.*, 2016). In line with these results, purified orfamide of *P. protegens* F6 was earlier found to cause insecticidal activity towards the aphid *Myzus persicae* (Jang *et al.*, 2013). In *Drosophila* infections by Pf-5, a lack of the macrolide molecule rhizoxin was found to cause a strong decrease of virulence. Moreover, rhizoxin was identified as the factor responsible for morphological defects observed in flies emerging from *P. protegens* Pf-5 infected larvae (Loper *et al.*, 2016). Genes related to those encoding Tc's in *Photorhabdus* and *Xenorhabdu*s spp. have been detected in several genomes of fluorescent pseudomonads and may have been acquired by horizontal gene transfer (Loper *et al.*, 2012). However, presence of Tc genes does not correlate with insecticidal activity of the respective strains and their role in insecticidal activity is still unclear (Rangel *et al.*, 2016).

For full virulence of *P. protegens* CHA0 specific cell surface components were found to be essential (Kupferschmied *et al.*, 2016). The outer membrane of Gram-negative bacteria consists mainly of lipopolysaccharides, which consist of a conserved lipid A-core oligosaccharide and a highly variable O-antigenic polysaccharide (O-PS). Mutants of *P. protegens* CHA0 lacking the dominant version of O-PS exhibited reduced virulence in injection and feeding experiments with *G. mellonella* and *P. xylostella*, respectively. Moreover, their resistance to insect antimicrobial peptides was

significantly reduced compared to the wild type. The specific O-PS might thus protect *P. protegens* CHA0 from the immune system of the insect (Kupferschmied *et al.*, 2016). Furthermore, certain exopolysaccharides and the type VI secretion system seem to play a role in insecticidal activity of *P. protegens* CHA0 (Keel, 2016).

Recently, by a screening of soil-isolated microbial strains a *P. chlororaphis* strain producing a proteinaceous insect toxin was discovered (Schellenberger *et al.*, 2016). The toxin IPD072Aa was found to exhibit insecticidal activity against the western corn rootworm, *Diabrotica virgifera virgifera*, but did not cause mortality in several lepidopteran and hemipteran insect species. The toxin gene was expressed in corn plants and proofed to confer resistance against the western corn rootworm under field conditions (Schellenberger *et al.*, 2016).

1.11. Ecological considerations of *Pseudomonas*-insect associations

While plant-beneficial traits and more recently also insecticidal activity of fluorescent pseudomonads raised attention because of potential applications as biocontrol agents, the different life-styles of these bacteria are also very interesting in a context of ecology and evolution.

Pseudomonas bacteria are ecologically very versatile, inhabiting various habitats such as plant-surfaces, animals, soil and water. Many strains that have been studied so far in a specific context or host might be much more ubiquitous. For example the insect pathogen *P. entomophila* was found to exhibit biocontrol effects against plant-pathogens (Vallet-Gely *et al.*, 2010a) and the plant-pathogen *P.* syringae was brought in the context of global water cycles (Morris *et al.*, 2008; Morris *et al.*, 2013). Furthermore, several strains belonging to the *P. fluorescens* group were recently isolated from water animals such as salmon eggs, perch intestine and cyclops (Burr *et al.*, 2010; Liu *et al.*, 2015; Ruffner *et al.*, 2015), though, their role in these hosts is still elusive.

Also the ecological relevance of insect-associations of fluorescent pseudomonads is largely unknown. In many studies *Pseudomonas* species were found in the digestive tract of insects (Kupferschmied *et al.*, 2013). These might acquire *Pseudomonas* bacteria from the environment accidentally via feeding on colonized plant material. Still, the specific adaptation of certain fluorescent pseudomonads discussed above, indicate an association with insect hosts of more than just coincidence. However, to what extent *P.*

protegens and *P. chlororaphis* are able to establish in wild insect populations and whether they can persist in insect hosts throughout different life-stages, i.e. from larva to pupa to adult, a phenomenon called transstadial transmission, remains to be investigated. For *P. syringae* several studies report transmission via insects to new host plants (Nadarasah and Stavrinides, 2011), a function for insect hosts, which is also conceivable in case of *P. protegens* and *P. chlororaphis*.

First results indicate that *P. protegens* CHA0 exhibits some degree of host specificity towards certain groups of insects. While it caused lethal infections in larvae of several lepidopteran species, it for instance did not cause significant mortality in an experiment with bumblebees (Ruffner, 2013). Nevertheless, the host range in nature remains speculative and it is unclear to what extent *P. protegens* and *P. chlororaphis* are able to cause lethal infections in healthy insect populations living in natural environments. These questions, however, are of great importance especially if envisaging an application of these bacteria as insect biocontrol agents.

1.12. Aims of the thesis and thesis outline

The discovery of insecticidal activity in root-colonizing plant-beneficial pseudomonads raised many questions about the mechanisms these bacteria use to infect insect hosts, about the ecological implications of the ability to switch between the two life styles and about possible exploitations of their dual activity for pest control. The overall aim of this thesis was to shed light on these different aspects. In a first part of the project we aimed at identifying virulence factors contributing to insecticidal activity. The focus in a second part was on the insect as an alternative host with a focus on bacterial survival throughout the insect life-cycle and the possibility of insect mediated dispersal. Finally, in a third part we investigated phylogenetic distribution of plant beneficial and insecticidal activity throughout the *P. fluorescens* group to identify strains with potent dual activity and we tested for the first time whether an insecticidal strain exhibits biocontrol activity in planta against root-feeding insect pests.

1. *Identification of virulence factors:*

   At the beginning of this thesis, the only known virulence factor involved in insecticidal activity of plant-beneficial pseudomonads was the Fit toxin. However, given the fact that a Fit deficient mutant retains considerable virulence and double mutant, lacking Fit and GacA, of *P. protegens* CHA0 is almost avirulent, the existence of further toxicity factors, at least partly regulated by the GacS/GacA system, seemed obvious. Therefore, we aimed at identifying additional factors contributing to insecticidal activity by following two approaches. On one hand we hypothesized that Gac regulated secondary metabolites and enzymes important for biocontrol against phytopathogenic fungi might be also involved in insecticidal activity. Therefore, we tested a collection of available and newly generated mutants of three *Pseudomonas* strains deficient for one or several metabolites in injection experiments with *G. mellonella* larvae as well as in a for this purpose developed oral test system with *P. xylostella* larvae. This approach revealed that several cyclic lipopeptides and hydrogen cyanide contribute to insecticidal activity of strains of the *P. chlororaphis* subgroup **(Chapter 3)**. On the other hand, we assumed that insecticidal strains share traits enabling insect infections that are absent in non-insecticidal strains. Therefore, we tested 26 strains of the *P. fluorescens* group for injectable and oral insecticidal activity and identified the *P. chlororaphis* subgroup

as highly insecticidal and the *P. fluorescens* subgroup as moderately insecticidal while strains of other tested subgroups exhibited no insecticidal activity. We sequenced the complete genomes of the investigated strains and by comparative genomics identified a set of almost 200 genes to be common and unique to insecticidal strains, thus, representing promising potential toxicity factors. By mutation of four of the candidate genes/ gene clusters we discovered two more factors contributing to insecticidal activity. Taken together the two approaches remarkably increased our understanding of the mechanism underlying insecticidal activity of fluorescent pseudomonads. In addition, the generated genomic data, including that of several species type strains, allowed phylogenetic reclassification of several strains and provides an invaluable resource for future investigations on any trait exhibited by *P. fluorescens* group bacteria **(Chapter 2)**.

2. *The insect as alternative host and a means for dispersal*

The discovery of an insect toxin in plant-beneficial pseudomonads imposes the question about the significance of insect associations in the ecology of these bacteria. Bacteria of the genus *Pseudomonas* are widely found as members of the natural mid-gut flora of insects and herbivorous insects feeding on plant material might accidentally take up plant-associated pseudomonads. However, the specific adaptations of strains of the *P. chlororaphis* subgroup indicate a more particular association with insects. We were therefore wondering how the bacteria behave and utilize the insect as an alternative host in lethal and sublethal infections. We were especially interested whether the bacteria persist in the insect and are transstadially transmitted. Moreover, dispersal abilities of bacteria on their own are rather limited and an insect host might be a welcome vehicle to overcome large distances and conquer new habitats. We found that *P. protegens* CHA0 can colonize larvae of different insect orders and in several cases is able to persist throughout metamorphosis, being transmitted to the pupal and partially even to the adult stage **(Chapter 4)**. We established a microscopy method to track the bacteria during the infection of *P. xylostella* larvae and to study in the same insect possible histopathological changes induced in the insect host **(Chapter 4)**. Furthermore, we provide evidence that *P. protegens* CHA0 taken up from roots by the root feeding insect pest *D. radicum* can persist and finally be transferred by the emerging flies to

a new plant, the roots of which as a consequence are again colonized by CHA0 **(Chapter 4)**. Besides persistence of CHA0 in sublethal infections we also identified several non-insecticidal strains of the *P. fluorescens* group to be able to survive and colonize *S. littoralis* larvae **(Chapter 2)**. Thus, although in certain cases *P. fluorescens* group bacteria rapidly infect and kill insect larvae, in other cases they exhibit long-term persistence over different stages of the insect life cycle using the insect as a habitat and potential means of dispersal.

3. *Biocontrol activity against root-feeding insects*

By the start of this project oral insecticidal activity of *P. protegens* and *P. chlororaphis* had only been demonstrated in artificial test systems, e.g. in Petri dish-assays with artificial diet or detached leaves, and only against insects feeding on leaves or rotting fruits (Ruffner *et al.*, 2013). A major aim of this project was to assess the biocontrol activity of insecticidal *Pseudomonas* strains in planta and specifically against root feeding insects. We therefore developed a test system with the cabbage root fly, *Delia radicum*, infesting cauliflower roots. Although survival of larvae grown on roots inoculated with *P. protegens* CHA0 was not significantly reduced compared to the control we observed morphological defects in parts of the emerging flies. Despite no direct biocontrol effect on the larvae at the tested bacterial concentrations the treatment with *P. protegens* CHA0 hence seems to affect insect fitness and could thus reduce population size of the pest **(Chapter 4)**.

Since many fluorescent pseudomonads exhibit excellent antifungal properties a combined application against a dual attack by a soil-borne fungal pathogen and an insect root pest could be envisaged. Therefore, we tested our collection of 26 strains mentioned above not only for insecticidal activity, but also for inhibition of two important soil-borne diseases. This way we identified a set of strains with potent dual activity **(Chapter 2)**.

1.13. References

Agrios G. N. (2005). *Plant Pathology, 5th edition*. Elsevier Academic Press: London, UK.

Appel H. M., Martin M. M. (1990). Gut redox conditions in herbivorous lepidopteran larvae. *J Chem Ecol* 16**:** 3277-3290.

Avet-Rochex A., Bergeret E., Attree I., Meister M., Fauvarque M. O. (2005). Suppression of *Drosophila* cellular immunity by directed expression of the ExoS toxin GAP domain of *Pseudomonas aeruginosa*. *Cell Microbiol* 7**:** 799-810.

Bakker P. A. H. M., Pieterse C. M. J., van Loon L. C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology* 97**:** 239-243.

Bale J. S., van Lenteren J. C., Bigler F. (2008). Biological control and sustainable food production. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 363**:** 761-776.

Berendsen R. L., Pieterse C. M. J., Bakker P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci* 17**:** 478-486.

Berg G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 84**:** 11-18.

Blackburn M. B., Martin P. A., Kuhar D., Farrar R. R., Jr., Gundersen-Rindal D. E. (2011). The occurrence of *Photorhabdus*-like toxin complexes in *Bacillus thuringiensis*. *PloS one* 6**:** e18122.

Blom J., Albaum S. P., Doppmeier D., Puehler A., Vorhoelter F.-J., Zakrzewski M. *et al.* (2009). EDGAR: A software framework for the comparative analysis of prokaryotic genomes. *BMC Bioinformatics* 10**:** 154.

Blom J., Kreis J., Spanig S., Juhre T., Bertelli C., Ernst C. *et al.* (2016). EDGAR 2.0: an enhanced software platform for comparative gene content analyses. *Nucleic Acids Res* 44**:** W22-W28.

Borlee B. R., Geske G. D., Robinson C. J., Blackwell H. E., Handelsman J. (2008). Quorum-sensing signals in the microbial community of the cabbage white butterfly larval midgut. *The ISME journal* 2**:** 1101-1111.

Bosshard A. (2016). *Pestizid-Reduktionsplan Schweiz*. Vision Landwirtschaft: Oberwil-Lieli, Switzerland.

Broderick K. E., Chan A., Balasubramanian M., Feala J., Reed S. L., Panda M. *et al.* (2008). Cyanide produced by human isolates of *Pseudomonas aeruginosa* contributes to lethality in *Drosophila melanogaster*. *J Infect Dis* 197**:** 457-464.

Buchon N., Broderick N. A., Lemaitre B. (2013). Gut homeostasis in a microbial world: insights from *Drosophila melanogaster*. *Nat Rev Microbiol* 11**:** 615-626.

Burr S. E., Gobeli S., Kuhnert P., Goldschmidt-Clermont E., Frey J. (2010). *Pseudomonas chlororaphis* subsp. *piscium* subsp. nov., isolated from freshwater fish. *Int J Syst Evol Microbiol* 60**:** 2753-2757.

Chakrabarti S., Liehl P., Buchon N., Lemaitre B. (2012). Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the *Drosophila* gut. *Cell host & microbe* 12**:** 60-70.

Chakroun M., Banyuls N., Bel Y., Escriche B., Ferre J. (2016). Bacterial vegetative insecticidal proteins (Vip) from entomopathogenic bacteria. *Microbiol Mol Biol Rev* 80**:** 329-350.

Chapman R. F. (1998). Alimentary canal, digestion and absorption. *The insects: structure and function*, 4 edn. Cambridge University Press: New York, USA. pp 38-68.

Chieda Y., Iiyama K., Yasunaga-Aoki C., Lee J. M., Kusakabe T., Shimizu S. (2005). Pathogenicity of *gacA* mutant of *Pseudomonas aeruginosa* PA01 in the silkworm, *Bombyx mori*. *FEMS Microbiol Lett* 244**:** 181-186.

Chieda Y., Iiyama K., Lee J. M., Kusakabe T., Yasunaga-Aoki C., Shimizu S. (2011). Virulence of an exotoxin A-deficient strain of *Pseudomonas aeruginosa* toward the silkworm, *Bombyx mori*. *Microb Pathog* 51**:** 407-414.

Chugani S. A., Whiteley M., Lee K. M., D'Argenio D., Manoil C., Greenberg E. P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* 98**:** 2752-2757.

Crickmore N. (2016). *Bacillus thuringiensis* resistance in *Plutella* - too many trees? *Curr Opin Insect Sci* 15**:** 84-88.

Daborn P. J. (2002). A single *Photorhabdus* gene, *makes caterpillars floppy* (*mcf*), allows *Escherichia coli* to persist within and kill insects. *Proceedings of the National Academy of Sciences* 99**:** 10742-10747.

De Vleesschauwer D., Höfte M. (2009). Rhizobacteria-induced systemic resistance. *Adv Bot Res* 51**:** 223-281.

Dieppois G., Opota O., Lalucat J., Lemaitre B. (2015). *Pseudomonas entomophila*: A versatile bacterium with enthomopathogenic properties. In: Ramos J.-L., Goldberg J. B., Filloux A. (eds). *Pseudomonas, Volume 7: New Aspects of Pseudmonas Biology*. vol. 7. Springer: Heidelberg, Germany. pp 25-49.

Ehlers R.-U. (2011). Regulation of biological control agents and the EU policy support action REBECA. In: Ehlers R.-U. (ed). *Regulation of biological control agents*. Springer: Dordrecht, Netherlands. pp 3-23.

Eilenberg J., Hajek A., Lomer C. (2001). Suggestions for unifying the terminology in biological control. *BioControl* 46**:** 387-400.

Engel P., Moran N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev* 37**:** 699-735.

Fairbrother A., Purdy J., Anderson T., Fell R. (2014). Risks of neonicotinoid insecticides to honeybees. *Environ Toxicol Chem* 33**:** 719-731.

Fauvarque M. O., Bergeret E., Chabert J., Dacheux D., Satre M., Attree I. (2002). Role and activation of type III secretion system genes in *Pseudomonas aeruginosa*-induced *Drosophila* killing. *Microb Pathog* 32**:** 287-295.

ffrench-Constant R. H., Dowling A., Waterfield N. R. (2007). Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon* 49**:** 436-451.

Godfray H. C. J., Beddington J. R., Crute I. R., Haddad L., Lawrence D., Muir J. F. *et al.* (2010). Food security: the challenge of feeding 9 billion people. *Science* 327**:** 812-818.

Gross H., Loper J. E. (2009). Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat Prod Rep* 26**:** 1408-1446.

Gross M. (2013). EU ban puts spotlight on complex effects of neonicotinoids. *Curr Biol* 23**:** R462-R464.

Haas D., Keel C. (2003). Regulation of antibiotic production in root-colonizing *Peudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol* 41**:** 117-153.

Haas D., Défago G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 3**:** 307-319.

Haloi K., Kalita M. K., Nath R., Devi D. (2016). Characterization and pathogenicity assessment of gut-associated microbes of muga silkworm A*ntheraea assamensis* Helfer (Lepidoptera: Saturniidae). *J Invertebr Pathol* 138**:** 73-85.

Hares M. C., Hinchliffe S. J., Strong P. C., Eleftherianos I., Dowling A. J., ffrench-Constant R. H. *et al.* (2008). The *Yersinia pseudotuberculosis* and *Yersinia pestis* toxin complex is active against cultured mammalian cells. *Microbiology* 154**:** 3503-3517.

Harrison J. F. (2001). Insect acid-base physiology. *Annu Rev Entomol* 46**:** 221-250.

Harrison R., Hoover K. (2012). Baculoviruses and other occluded insect viruses. In: Vega F. E., Kaya H. K. (eds). *Insect pathology*, 2 edn. Elsevier: London, UK. pp 73-131.

Hilker R., Munder A., Klockgether J., Losada P. M., Chouvarine P., Cramer N. *et al.* (2015). Interclonal gradient of virulence in the *Pseudomonas aeruginosa* pangenome from disease and environment. *Environ Microbiol* 17**:** 29-46.

Hurst M. R., Glare T. R., Jackson T. A. (2004). Cloning *Serratia entomophila* antifeeding genes–a putative defective prophage active against the grass grub *Costelytra zealandica*. *J Bacteriol* 186**:** 5116-5128.

Iavicoli A., Boutet E., Buchala A., Metraux J. P. (2003). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Mol Plant-Microbe Interact* 16**:** 851-858.

Iiyama K., Chieda Y., Lee J. M., Kusakabe T., Yasunaga-Aoki C., Shimizu S. (2007). Effect of superoxide dismutase gene inactivation on virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm, *Bombyx mori*. *Appl Environ Microbiol* 73**:** 1569-1575.

Jang J. Y., Yang S. Y., Kim Y. C., Lee C. W., Park M. S., Kim J. C. *et al.* (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J Agric Food Chem* 61**:** 6786-6791.

Kamareddine L. (2012). The biological control of the malaria vector. *Toxins* 4**:** 748-767.

Keel C., Voisard C., Berling C. H., Kahr G., Défago G. (1989). Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* 79**:** 584-589.

Keel C., Wirthner P., Oberhansli T., Voisard C., Burger, Haas D. *et al.* (1990). Pseudomonads as antagonists of plant pathogens in the rhizosphere - role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. *Symbiosis* 9**:** 327-341.

Keel C., Schnider U., Maurhofer M., Voisard C., Laville J., Burger U. *et al.* (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0 - Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant-Microbe Interact* 5**:** 4-13.

Keel C. (2016). A look into the toolbox of multi-talents: insect pathogenicity determinants of plant-beneficial pseudomonads. *Environ Microbiol* 18**:** 3207-3209.

Kupferschmied P., Maurhofer M., Keel C. (2013). Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Frontiers in plant science* 4**:** 287.

Kupferschmied P., Péchy-Tarr M., Imperiali N., Maurhofer M., Keel C. (2014). Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. *PLoS Path* 10**:** e1003964.

Kupferschmied P. (2015). Molecular basis and regulation of insect pathogenicity in plant-beneficial pseudmonads. PhD thesis, University of Lausanne.

Kupferschmied P., Chai T., Flury P., Blom J., Smits T. H., Maurhofer M. *et al.* (2016). Specific surface glycan decorations enable antimicrobial peptide resistance in plant-beneficial

pseudomonads with insect-pathogenic properties. *Environ Microbiol* [Epub ahead of print].

Lacey L. A., Grzywacz D., Shapiro-Ilan D. I., Frutos R., Brownbridge M., Goettel M. S. (2015). Insect pathogens as biological control agents: Back to the future. *J Invertebr Pathol* 132**:** 1-41.

Laville J., Voisard C., Keel C., Maurhofer M., Defago G., Haas D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings of the National Academy of Sciences of the United States of America* 89**:** 1562-1566.

Lehane M. J. (1997). Peritrophic matrix structure and function. *Annu Rev Entomol* 42**:** 525-550.

Lemaitre B., Hoffmann J. (2007). The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 25**:** 697-743.

Lewis E. E., Clarke D. J. (2012). Nematode parasites and entomopathogens. In: Vega F. E., Kaya H. K. (eds). *Insect Pathology*, 2 edn. Elsevier: London, UK. pp 395-424.

Liehl P., Blight M., Vodovar N., Boccard F., Lemaitre B. (2006). Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. *PLoS Path* 2**:** e56.

Limmer S., Haller S., Drenkard E., Lee J., Yu S., Kocks C. *et al.* (2011). *Pseudomonas aeruginosa* RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model. *Proceedings of the National Academy of Sciences of the United States of America* 108**:** 17378-17383.

Liu J. R., Lin Y. D., Chang S. T., Zeng Y. F., Wang S. L. (2010). Molecular cloning and characterization of an insecticidal toxin from *Pseudomonas taiwanensis*. *J Agric Food Chem* 58**:** 12343-12349.

Liu Y., Rzeszutek E., van der Voort M., Wu C. H., Thoen E., Skaar I. *et al.* (2015). Diversity of aquatic *Pseudomonas* species and their activity against the fish pathogenic oomycete *Saprolegnia*. *PloS one* 10**:** e0136241.

Loper J. E., Hassan K. A., Mavrodi D. V., Davis E. W., 2nd, Lim C. K., Shaffer B. T. *et al.* (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet* 8**:** e1002784.

Loper J. E., Henkels M. D., Rangel L. I., Olcott M. H., Walker F. L., Bond K. L. *et al.* (2016). Rhizoxin, orfamide A, and chitinase production contribute to the toxicity of *Pseudomonas protegens* strain Pf-5 to *Drosophila melanogaster*. *Environ Microbiol* 18**:** 3509-3521.

Lugtenberg B., Kamilova F. (2009). Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* 63**:** 541-556.

Lugtenberg B. J., Malfanova N., Kamilova F., Berg G. (2013a). Microbial control of plant root diseases. In: de Bruijn F. J. (ed). *Molecular microbial ecology of the rhizosphere*. vol. 2. John Wiley & Sons, Inc: Hoboken, New Jersey. pp 575-586.

Lugtenberg B. J., Malfanova N., Kamilova F., Berg G. (2013b). Plant growth promotion by microbes. In: de Bruijn F. J. (ed). *Molecular microbial ecology of the rhizosphere*. vol. 2. John Wiley & Sons, Inc: Hoboken, New Jersey. pp 561-573.

Maurhofer M., Keel C., Schnider U., Voisard C., Haas D., Defago G. (1992). Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology* 82**:** 190-195.

Maurhofer M., Hase C., Meuwly P., Metraux J. P., Defago G. (1994). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas*

fluorescens strain CHA0 - Influence of the *GacA* gene and of pyoverdine production. *Phytopathology* 84**:** 139-146.

Meister M. (2004). Blood cells of *Drosophila*: cell lineages and role in host defence. *Curr Opin Immunol* 16**:** 10-15.

Mendes R., Kruijt M., de Bruijn I., Dekkers E., van der Voort M., Schneider J. H. *et al.* (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332**:** 1097-1100.

Mercado-Blanco J., Bakker P. A. H. M. (2007). Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Anton Leeuw Int J G* 92**:** 367-389.

Mercado-Blanco J. (2015). *Pseudomonas* strains that exert biocontrol of plant pathogens. In: Ramos J.-L., Goldberg J. B., Filloux A. (eds). *Pseudomonas, Volume 7: New Aspects of Pseudomonas Biology*. vol. 7. Springer Heidelberg, Germany. pp 121-172.

Meyer J. B., Frapolli M., Keel C., Maurhofer M. (2011). Pyrroloquinoline quinone biosynthesis gene *pqqC*, a novel molecular marker for studying the phylogeny and diversity of phosphate-solubilizing pseudomonads. *Appl Environ Microbiol* 77**:** 7345-7354.

Miyata S., Casey M., Frank D. W., Ausubel F. M., Drenkard E. (2003). Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* 71**:** 2404-2413.

Morris C. E., Sands D. C., Vinatzer B. A., Glaux C., Guilbaud C., Buffiere A. *et al.* (2008). The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME journal* 2**:** 321-334.

Morris C. E., Monteil C. L., Berge O. (2013). The life history of *Pseudomonas syringae*: linking agriculture to earth system processes. *Annu Rev Phytopathol* 51**:** 85-104.

Nadarasah G., Stavrinides J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol Rev* 35**:** 555-575.

Oerke E. C. (2005). Crop losses to pests. *The Journal of Agricultural Science* 144**:** 31-43.

Okuda J., Hayashi N., Okamoto M., Sawada S., Minagawa S., Yano Y. *et al.* (2010). Translocation of *Pseudomonas aeruginosa* from the intestinal tract is mediated by the binding of ExoS to an Na,K-ATPase regulator, FXYD3. *Infect Immun* 78**:** 4511-4522.

Olcott M. H., Henkels M. D., Rosen K. L., Walker F. L., Sneh B., Loper J. E. *et al.* (2010). Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. *PloS one* 5**:** e12504.

Opota O., Vallet-Gely I., Vincentelli R., Kellenberger C., Iacovache I., Gonzalez M. R. *et al.* (2011). Monalysin, a novel -pore-forming toxin from the *Drosophila* pathogen *Pseudomonas entomophila*, contributes to host intestinal damage and lethality. *PLoS Path* 7**:** e1002259.

Osborn F., Berlioz L., Vitelli-Flores J., Monsalve W., Dorta B., Lemoine V. R. (2002). Pathogenic effects of bacteria isolated from larvae of *Hylesia metabus* Crammer (Lepidoptera: Saturniidae). *J Invertebr Pathol* 80**:** 7-12.

Palma L., Munoz D., Berry C., Murillo J., Caballero P. (2014). *Bacillus thuringiensis* toxins: an overview of their biocidal activity. *Toxins* 6**:** 3296-3325.

Parnell J. J., Berka R., Young H. A., Sturino J. M., Kang Y., Barnhart D. M. *et al.* (2016). From the lab to the farm: an industrial perspective of plant beneficial microorganisms. *Frontiers in plant science* 7**:** 1110.

Paulsen I. T., Press C. M., Ravel J., Kobayashi D. Y., Myers G. S., Mavrodi D. V. *et al.* (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol* 23**:** 873-878.

Péchy-Tarr M., Bruck D. J., Maurhofer M., Fischer E., Vogne C., Henkels M. D. *et al.* (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ Microbiol* 10**:** 2368-2386.

Péchy-Tarr M., Borel N., Kupferschmied P., Turner V., Binggeli O., Radovanovic D. *et al.* (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ Microbiol* 15**:** 736-750.

Pineda A., Zheng S. J., van Loon J. J., Pieterse C. M., Dicke M. (2010). Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends Plant Sci* 15**:** 507-514.

Raaijmakers J. M., Vlami M., de Souza J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Anton Leeuw Int J G* 81**:** 537-547.

Raaijmakers J. M., Paulitz T. C., Steinberg C., Alabouvette C., Moënne-Loccoz Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321**:** 341-361.

Raaijmakers J. M., Mazzola M. (2016). Soil immune responses: soil microbiomes may be harnessed for plant health. *Science* 352**:** 1392-1393.

Ramette A., Frapolli M., Fischer-Le Saux M., Gruffaz C., Meyer J.-M., Défago G. *et al.* (2011). *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. *Syst Appl Microbiol* 34**:** 180-188.

Rangel L. I., Henkels M. D., Shaffer B. T., Walker F. L., Davis E. W., 2nd, Stockwell V. O. *et al.* (2016). Characterization of toxin complex gene clusters and insect toxicity of bacteria representing four subgroups of *Pseudomonas fluorescens*. *PloS one* 11**:** e0161120.

Roy H. E., Steinkraus D. C., Eilenberg J., Hajek A. E., Pell J. K. (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annu Rev Entomol* 51**:** 331-357.

Ruffner B. (2013). Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis, ETH Zurich, Zurich, Switzerland.

Ruffner B., Péchy-Tarr M., Ryffel F., Hoegger P., Obrist C., Rindlisbacher A. *et al.* (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ Microbiol* 15**:** 751-763.

Ruffner B., Pechy-Tarr M., Hofte M., Bloemberg G., Grunder J., Keel C. *et al.* (2015). Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* 16**:** 609.

Saravanakumar D., Muthumeena K., Lavanya N., Suresh S., Rajendran L., Raguchander T. *et al.* (2007). *Pseudomonas*-induced defence molecules in rice plants against leaffolder (*Cnaphalocrocis medinalis*) pest. *Pest Manag Sci* 63**:** 714-721.

Schellenberger U., Oral J., Rosen B. A., Wei J.-Z., Zhu G., Xie W. *et al.* (2016). A selective insecticidal protein from *Pseudomonas* for controlling corn rootworms. *Science* [Epub ahead of print].

Shapiro-Ilan D. I., Bruck D., Lacey L. A. (2012). Principles of epizootiology and microbial control. In: Vega F. E., Kaya H. K. (eds). *Insect Pathology*, 2 edn. Elsevier: London, UK. pp 29-72.

Simon-Delso N., Amaral-Rogers V., Belzunces L. P., Bonmatin J. M., Chagnon M., Downs C. *et al.* (2015). Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental science and pollution research international* 22**:** 5-34.

Stavrinides J., McCloskey J. K., Ochman H. (2009). Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. *Appl Environ Microbiol* 75**:** 2230-2235.

Stockwell V. O., Stack J. P. (2007). Using *Pseudomonas* spp. for integrated biological control. *Phytopathology* 97**:** 244-249.

Stutz E. W., Défago G., Kern H. (1986). Naturally-occuring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* 76**:** 181-185.

Tabashnik B. E., Brevault T., Carriere Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. *Nat Biotechnol* 31**:** 510-521.

Vallet-Gely I., Lemaitre B., Boccard F. (2008). Bacterial strategies to overcome insect defences. *Nat Rev Microbiol* 6**:** 302-313.

Vallet-Gely I., Novikov A., Augusto L., Liehl P., Bolbach G., Pechy-Tarr M. *et al.* (2010a). Association of hemolytic activity of *Pseudomonas entomophila*, a versatile soil bacterium, with cyclic lipopeptide production. *Appl Environ Microbiol* 76**:** 910-921.

Vallet-Gely I., Opota O., Boniface A., Novikov A., Lemaitre B. (2010b). A secondary metabolite acting as a signalling molecule controls *Pseudomonas entomophila* virulence. *Cell Microbiol* 12**:** 1666-1679.

van de Mortel J. E., de Vos R. C. H., Dekkers E., Pineda A., Guillod L., Bouwmeester K. *et al.* (2012). Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiol* 160**:** 2173-2188.

van der Sluijs J. P., Simon-Delso N., Goulson D., Maxim L., Bonmatin J. M., Belzunces L. P. (2013). Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr Opin Env Sust* 5**:** 293-305.

Van Oosten V. R., Bodenhausen N., Reymond P., Van Pelt J. A., Van Loon L. C., Dicke M. *et al.* (2008). Differential effectiveness of microbially induced resistance against herbivorous insects in *Arabidopsis*. *Mol Plant-Microbe Interact* 21**:** 919-930.

Vodovar N., Vinals M., Liehl P., Basset A., Degrouard J., Spellman P. *et al.* (2005). *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. *Proceedings of the National Academy of Sciences of the United States of America* 102**:** 11414-11419.

Vodovar N., Vallenet D., Cruveiller S., Rouy Z., Barbe V., Acosta C. *et al.* (2006). Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. *Nat Biotechnol* 24**:** 673-679.

Voisard C., Keel C., Haas D., Defago G. (1989). Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *EMBO J* 8**:** 351-358.

Waterfield N. R., Daborn P. J., Dowling A. J., Yang G., Hares M., ffrench-Constant R. H. (2003). The insecticidal toxin Makes caterpillars floppy 2 (Mcf2) shows similarity to HrmA, an avirulence protein from a plant pathogen. *FEMS Microbiol Lett* 229**:** 265-270.

Weller D. M., Raaijmakers J. M., Gardener B. B., Thomashow L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol* 40**:** 309-348.

Weller D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* 97**:** 250-256.

CHAPTER 2

Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics

This chapter was published in *The ISME Journal* (2016) 10: 2527–2542, by

Pascale Flury[1], Nora Aellen[1], Beat Ruffner[1], Maria Péchy-Tarr[2], Shakira Fataar[1], Zane Metla[1,3], Ana Dominguez-Ferreras[1], Guido Bloemberg[4], Joachim Frey[5], Alexander Goesmann[6], Jos M. Raaijmakers[7], Brion Duffy[8], Monica Höfte[9], Jochen Blom[6], Theo H. M. Smits[8]*, Christoph Keel[2]* and Monika Maurhofer[1]*

[1]Plant Pathology, Institute of Integrative Biology, ETH Zürich, Zürich, Switzerland
[2]Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland
[3]Laboratory of Experimental Entomology, Institute of Biology, University of Latvia, Latvia
[4]Institute of Medical Microbiology, University of Zurich, Zürich, Switzerland
[5]Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland
[6]Bioinformatics and Systems Biology, Justus-Liebig-University Giessen, Giessen, Germany
[7]Department of Microbial Ecology, Netherlands Institute of Ecology, NIOO-KNAW, 6708 PB, Wageningen, Netherlands
[8]Environmental Genomics and Systems Biology Research Group, Institute for Natural Resource Sciences, Zürich University of Applied Sciences, Wädenswil, Switzerland
[9]Laboratory of Phytopathology, Faculty of Bioscience Engineering, Ghent University, B-9000 Ghent, Belgium
*corresponding authors

ABSTRACT

Bacteria of the genus *Pseudomonas* occupy diverse environments. The *Pseudomonas fluorescens* group is particularly well-known for its plant-beneficial properties including pathogen suppression. Recent observations that some strains of this group also cause lethal infections in insect larvae, however, point to a more versatile ecology of these bacteria. We show that 26 *P. fluorescens* group strains, isolated from three continents and covering three phylogenetically distinct sub-clades, exhibited different activities towards lepidopteran larvae, ranging from lethal to avirulent. All strains of sub-clade 1, which includes *Pseudomonas chlororaphis* and *Pseudomonas protegens*, were highly insecticidal regardless of their origin (animals, plants). Comparative genomics revealed that strains in this sub-clade possess specific traits allowing a switch between plant and insect-associated lifestyles. We identified 90 genes unique to all highly insecticidal strains (sub-clade 1) and 117 genes common to all strains of sub-clade 1 and present in some moderately insecticidal strains of sub-clade 3. Mutational analysis of selected genes revealed the importance of chitinase C and phospholipase C in insect pathogenicity. The study provides insight into the genetic basis and phylogenetic distribution of traits defining insecticidal activity in plant-beneficial pseudomonads. Strains with potent dual activity against plant pathogens and herbivorous insects have great potential for use in integrated pest management for crops.

INTRODUCTION

Bacteria of the genus *Pseudomonas* occupy diverse terrestrial, aquatic and atmospheric environments, exhibiting a wide variety of ecological behaviors. Some are feared as human or plant pathogens such as *Pseudomonas aeruginosa* or *Pseudomonas syringae;* others are welcome agents for bioremediation of pollutants such as *Pseudomonas putida.* Members of the *Pseudomonas fluorescens* group are well–known for plant-beneficial effects that improve crop health and agricultural production. Many strains of fluorescent pseudomonads isolated from the rhizosphere have been studied for their ability to suppress root diseases, to promote plant growth and to induce systemic resistance (Haas and Défago, 2005; Bakker *et al.*, 2007). They harbor strain-specific arsenals of antifungal metabolites, which enable them to inhibit pathogen growth through direct antibiosis (Haas and Keel, 2003; Raaijmakers *et al.*, 2010). All these features make fluorescent pseudomonads interesting organisms for use as biofertilizers and biopesticides in sustainable agriculture and several products have been commercialized (Kupferschmied *et al.*, 2013). On top of plant-beneficial activity, genomics has revealed unexpected and broader ecological versatility for these bacteria (Paulsen *et al.*, 2005; Loper *et al.*, 2012). Three of the best-characterized biocontrol strains, *Pseudomonas protegens* strains CHA0 and Pf-5 and *Pseudomonas chlororaphis* PCL1391, were shown to have potent insecticidal activity (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013). When injected into the hemocoel of *Galleria mellonella* or *Manduca sexta* larvae, they rapidly multiply and cause larval death within a few hours (Péchy-Tarr *et al.*, 2008). Ecologically more relevant, these strains are also able to infect and kill insect larvae, such as *Drosophila melanogaster* and the agricultural pests *Spodoptera littoralis* or *Plutella xylostella*, after oral uptake (Olcott *et al.*, 2010; Ruffner *et al.*, 2013). Oral insecticidal activity is considered a rare trait amongst bacteria and requires specific mechanisms to cope with host immune responses and to breach the gut epithelium in order to access the hemocoel (Vallet-Gely *et al.*, 2008; Opota *et al.*, 2011; Herren and Lemaitre, 2012). How *P. protegens* and *P. chlororaphis* overcome these barriers remains unclear. However, an association with insecticidal activity has been demonstrated for a set of genes termed the *fit* genes (*P. fluorescens* insecticidal toxin) (Péchy-Tarr *et al.*, 2008; Péchy-Tarr *et al.*, 2013; Ruffner *et al.*, 2013). The unique virulence cassette harbours the *fitD* gene encoding the proteinaceous Fit toxin as well as regulatory genes and a type I secretion system (Péchy-Tarr *et al.*, 2008; Péchy-Tarr *et*

al., 2013; Kupferschmied *et al.*, 2014). Nevertheless, *fitD* deletion mutants retain substantial toxicity, indicating the existence of additional virulence factors (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013). Mutational analyses provide evidence that some of them are regulated by the global regulator GacA (Olcott *et al.*, 2010; Ruffner *et al.*, 2013).

Insecticidal activity is not universal to the *P. fluorescens* group. A survey by Ruffner *et al.* (2015) revealed that sub-clade 2 strains (Loper et al 2012) neither harbor *fit* genes nor have ability to kill *G. mellonella* larvae. In contrast, all tested *P. protegens* and *P. chlororaphis* strains, which represent the sub-clade 1 (Loper *et al.*, 2012), have both the toxin and injectable activity. Accordingly, *Pseudomonas* sp. strains Pf-01 and Q2-87 (formerly called *P. fluorescens* Pf-01 and Q2-87), both belonging to sub-clade 2, have no oral activity against larvae of *D. melanogaster* and several lepidopteran species, respectively (Olcott *et al.*, 2010; Ruffner *et al.*, 2013). Interestingly, *Pseudomonas* sp. SBW25 (formerly called *P. fluorescens* SBW25) of sub-clade 3, which does not harbor the *fit* genes, was shown to cause mortality and developmental delay in *D. melanogaster* larvae, but to a much lower extent than *P. protegens* Pf-5 (Olcott *et al.*, 2010).

The discovery of insecticidal activity in fluorescent pseudomonads raises diverse ecological and agronomic questions. What ecological advantage may be gained by this ability to switch from a plant to an insect environment? Can we use these pseudomonads as double–agents to fight both plant disease and insect pests? To date, our understanding of the interaction of plant-associated pseudomonads with insects is still very poor. Although large differences in their ability to infect insects were found between the strains investigated so far, no extensive data on frequency and distribution of insecticidal activity throughout the whole *P. fluorescens* group is available and individual strains with different phylogenetic background have never been compared directly. Moreover, the precise factors beyond the Fit toxin, which enable certain fluorescent pseudomonads to kill insects, and thereby to occupy a habitat alternative to plant roots, are still elusive. As a first step towards understanding the genomic features enabling insect pathogenicity we have taken an approach that combines bioassays with comparative genomics. We investigated twenty-six strains of fluorescent pseudomonads for their insecticidal activity and their biocontrol activity against root diseases. The strains included in our study are representative of the three phylogenetic sub-clades within the *P. fluorescens* group that harbor most plant-beneficial

pseudomonads and were isolated from root but also from non-root habitats. Strong oral activity was found for all strains belonging to the phylogenetic sub-clade 1, which showed potent dual activity against insects and plant pathogens. However, we identified also a second phylogenetic group, sub-clade 3, containing strains with lower insecticidal activity. The strains were sequenced and comparative genomics revealed around 200 genes that are common and unique to the insecticidal strains and we hypothesize that this specific set of genes may represent major evolutionary events towards insect pathogenicity of *Pseudomonas* spp*.* Finally, we present first results from testing the involvement of some of the newly identified putative virulence factors in insecticidal activity using a mutational approach.

MATERIALS AND METHODS

Bacterial strains

Strain names and origins are listed in Table 1. We use species names only for strains that cluster closely to a species type strain in the phylogenetic tree we created based on core genomes (Figure 1) and thus can clearly be assigned to a certain species. All other strains are referred to as Pseudomonas sp. For sequencing, strains were taken from our long-term strain storage kept at -80°C, or were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). If not otherwise stated bacterial cultures for bioassays and sequencing were grown in LB medium (Bertani, 1951) overnight on a rotary shaker (180 rpm) at 24°C. For bioassays cells were washed in sterile 0.9% NaCl. OD600 was measured and cells diluted to the desired concentration, while assuming that a cell suspension with an OD600 of 0.125 contains approximately 108 colony forming units (cfu) per ml.

Genome sequencing, assembly and comparative genomics

For sequencing the genomes, DNA was extracted from overnight cultures in LB using the Wizard Genomic DNA Purification Kit (Promega AG, Dübendorf, Switzerland). All genomes apart from *Pseudomonas* sp. CMR5c (for this see Supplementary Methods) were sequenced on an Illumina MiSeq (2 × 300 bp shotgun sequencing) at the Quantitative Genomics Facility (QGF) of the BioSystems Science and Engineering department (BSSE) of ETH Zürich located in Basel, Switzerland. Subsequently, the reads were *de novo* assembled using SeqMan NGen12 (DNASTAR, Madison, WI, USA) and further manually improved *in silico* using different subroutines of the Genomics Package of LASERGENE 12 (DNASTAR, Madison, WI, USA).

All genome sequences generated in this study and several database sequences that do not contain an annotation were automatically annotated in GenDB (Meyer *et al.*, 2003). The annotation of the genome of *P. chlororaphis* subsp. *piscium* PCL1391 was manually improved and the whole Genome Shotgun project was deposited at DDBJ/EMBL/GenBank. Genomes of all other sequenced strains were deposited without annotations at DDBJ/EMBL/GenBank. Accession numbers are indicated in Supplementary Table S1.

Comparative genomics was done using EDGAR (Blom *et al.*, 2009). Gene sets common to

certain strains, but absent in other strains were calculated with a cut-off of 70% amino-acid identity over 70% of the gene length (Smits *et al.*, 2010). For the phylogenetic tree of the core genomes, annotated assemblies and genomes from the public GenBank database (NCBI) were used. However, for quality reasons only genomes that consisted of less than 200 contigs and are thus classified as "high-quality draft genome sequences" by the NCBI, were included.

The phylogeny based on the core genome of all included strains was generated in EDGAR. The phylogenetic tree was created with the neighbor joining algorithm on a Kimura distance matrix as implemented in the PHYLIP package (Blom *et al.*, 2009). Due to the huge size of the core alignment and the long resulting calculation time for a tree, bootstrapping was not performed.

Insect assays

Injection assays with *G. mellonella* were performed with small adaptations as described by Péchy-Tarr *et al.* (2008). More information is placed in Supplementary Methods.

Feeding assays: Eggs of *P. xylostella* were obtained from Syngenta Crop Protection AG (Stein, Switzerland). General growth conditions for larvae before and during the experiments were 26°C, 60% humidity and a 16-h day, 8-h night cycle. Prior to experiments, boxes with larvae were placed at 18°C in the dark for 48 h. For virulence assays, 1-week-old larvae were exposed to 10 µl washed bacterial cells adjusted to the desired concentration or 0.9% NaCl (controls) on a pellet of modified insect diet (Gupta *et al.*, 2005; Ruffner *et al.*, 2013). To prevent injuries each larva was kept separately in 128-cell bioassay trays (Frontier Agricultural Sciences, Delaware, USA). Each treatment was tested on four replicates of eight larvae. Mortality was defined as the inability to react to poking.

Construction of deletion mutants of *P. protegens* CHA0

The *chiC*, *aprX* and *plcN* genes and the *rebB1-3* cluster of *P. protegens* CHA0 were deleted by an allelic replacement technique using the I-SceI system with the suicide vector pEMG (Martinez-Garcia and de Lorenzo, 2011) as detailed in previous work (Kupferschmied *et al.*, 2014)). To construct the pEMG-based plasmids, the 600-700-bp upstream and downstream regions flanking the genomic region to be deleted were amplified by PCR using the primer pairs specified in Supplementary Table S2. The

obtained fragments were digested with the relevant restriction enzymes (Supplementary Table S2) and cloned into pEMG via triple ligation. Constructs were verified by sequencing. The obtained suicide plasmids served then to generate the deletion mutants CHA5099 (Δ*chiC*), CHA5222 (Δ*aprX*), CHA5223 (Δ*plcN*), and CHA5221 (Δ*rebB1-3*) (Supplementary Table S2), using the I-SceI system with the expression plasmid pSW-2.

Chitinase activity assay

Chitinase activity was measured in supernatants of cultures grown for 48 h in LB shaking with a methylumbelliferone-based chitinase assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

Statistics

Data analysis was performed in R version 3.1.1. (http://www.r-project.org). Mortality rates of the insect toxicity tests with wild type strains were analyzed by multiple comparisons using Kruskal-Wallis adjusted by Bonferroni-Holm. LT_{50} values were estimated based on the generalized linear model using the MASS package in R (Venables and Ripley, 2002). To test for significant differences between *P. protegens* CHA0 and its mutant strains the Log-Rank test of the Survival package of R and the Student's t-test were used in insect toxicity test and chitinase activity assays, respectively.

Table 1. Strain information

Strain	Former Name	Geographic Origin	Habitat/ Host[a]	Biocontrol ability	Genome sequenced	References
***P. protegens* CHA0^T**	*P. fluorescens* CHA0^T	Switzerland	Tobacco	Cucumber-Pu, Tobacco-Tb, Wheat-Ggt, Tomato-Forl	Jousset *et al.* (2014)	Haas and Défago (2005); Keel *et al.* (1996); Ramette *et al.* (2011); Stutz *et al.* (1986)
***P. protegens* PGNR1**	*P. fluorescens* PGNR1	Ghana	Tobacco	Cucumber-Pu, Tomato-Forl	this study	Keel *et al.* (1996)
***P. protegens* BRIP**		Switzerland	Cyclops	ND	this study	Ruffner *et al.* (2015)
***P. protegens* K94.41**	*P. fluorescens* K94.41	Slovakia	Cucumber	Cucumber-Pu, Tomato-Forl	this study	Wang *et al.* (2001)
***P. protegens* PF**	*P. fluorescens* PF	Oklahoma, USA	Wheat leaves	Wheat-St	this study	Keel *et al.* (1996); Levy *et al.* (1992)
***Pseudomonas* sp. CMR5c**		Cameroon	Red cocoyam	Cocoyam-Pm	this study	Perneel *et al.* (2007)
***Pseudomonas* sp. CMR12a**		Cameroon	Red cocoyam	Cocoyam-Pm, Bean-Rs	this study	D'aes *et al.* (2011); Perneel *et al.* (2007)
***P. chlororaphis* subsp. *piscium* DSM 21509^T**		Lake of Neuchâtel, Switzerland	Intestine of European perch	ND	this study	Burr *et al.* (2010)
***P. chlororaphis* subsp. *piscium* PCL1391**		Spain	Tomato	Tomato-Forl	this study	Chin-A-Woeng *et al.* (1998)
***P. chlororaphis* subsp. *aureofaciens* LMG 1245^T**		Netherlands	River Clay	ND	this study	Kluyver (1956); Peix *et al.* (2007)
***P. chlororaphis* subsp. *aureofaciens* CD**		Switzerland	Cyclops (water)	ND	this study	Ruffner *et al.* (2015)
***P. chlororaphis* subsp. *chlororaphis* LMG 5004^T**		-	Contaminated plate	ND	this study	Peix *et al.* (2007)
***P. brassicacearum* TM1A3**	*P. fluorescens* TM1A3	Switzerland	Tomato	Cucumber-Pu, Cotton-Rs	this study	Fuchs and Defago (1991); Keel *et al.* (1996)
***P. thivervalensis* DSM 13194^T**		France	Rapeseed	ND	this study	Achouak *et al.* (2000)
***P. thivervalensis* PITR2**	*P. fluorescens* PITR2	Albenga,Italy	Wheat	Cucumber-Pu, Tomato-Forl	this study	Keel *et al.* (1996)
***P. kilonensis* P12**	*P. fluorescens* P12	Switzerland	Tobacco	Tobacco-Tb	this study	Keel *et al.* (1996)
***P. kilonensis* DSM 13647^T**		Germany	Agricultural soil	ND	this study	Sikorski *et al.* (2001)
***Pseudomonas* sp. Q12-87**	*P. fluorescens* Q12-87	Washington, USA	Wheat	Wheat-Ggt	this study	Keel *et al.* (1996)
***Pseudomonas* sp. P97.38**	*P. fluorescens* P97.38	Switzerland	Cucumber	Cucumber-Pu, Tomato-Forl	this study	Wang *et al.* (2001)
***P. corrugata* DSM 7228^T**		United Kingdom	Tomato stem	ND	this study	Scarlett *et al.* (1978)
***Pseudomonas* sp. Pf153**	*P. fluorescens* Pf153	Switzerland	Tobacco	Cucumber-Pu	this study	Fuchs *et al.* (2000)
***Pseudomonas* sp. P1.8**		Switzerland	Earthworm	ND	this study	Ruffner *et al.* (2015)
***Pseudomonas* sp. P1.31**		Switzerland	Woodlouse (dead)	ND	this study	Ruffner *et al.* (2015)
***P. fluorescens* DSM 50090^T**		United Kingdom	pre-filter tanks	ND	this study	Rhodes (1959)
***Pseudomonas* sp. MIACH**	*P. fluorescens* MIACH	Switzerland	Wheat	ND	this study	Meyer *et al.* (2011)
***Pseudomonas* sp. SS101**	*P. fluorescens* SS101	The Netherlands	Wheat	Cucumber-Pc, Tomato-Pi	Loper *et al.* (2012)	de Souza *et al.* (2003); Kruijt *et al.* (2009); Mazzola *et al.* (2007); Tran *et al.* (2007)

Abbreviations: Forl, *Fusarium oxysporum* f. sp. *radicis-lycopersici*; Ggt, *Gaeumannomyces graminis* var. *tritici*; ND, not documented; Pc, *Phytophthora capsici*; Pi, *Phytophthora infestans*; Pm, *Pythium myriotylum;* Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; St, *Septoria tritici*; Tb, *Thielaviopsis basicola*.

[a] Plant hosts: If not otherwise stated strains were isolated from roots or rhizosphere.

RESULTS AND DISCUSSION

Strain selection

To obtain an extensive overview of the occurrence of insecticidal activity within the *P. fluorescens* group, we selected 26 strains (Table 1). Many strains were isolated from roots and are well-known for their activity against plant pathogens, others were recently isolated from completely different habitats such as perch intestine and cyclops, e.g., strains *P. chlororaphis* subsp. *piscium* DSM 21509^T and *P. protegens* BRIP, respectively. Type strains were included in the study when considerable indications were present for close relationships of non-assigned strains to existing species.

The included strains, isolated on three different continents, belong to five subgroups within the *P. fluorescens* group (Supplementary Figure S1)(Mulet *et al.*, 2012; Gomila *et al.*, 2015), that are covered in the three sub-clades defined by Loper *et al.* (2012)(Figure 1): twelve strains representing sub-clade 1, eleven strains representing four known and five new species in sub-clade 2 (including the *P. corrugata, P. koreensis* and *P. jessenii* subgroups), and three strains in sub-clade 3 (Figure 1 and Supplementary Figure S1). A detailed overview of the phylogeny of the included strains is given in Supplementary Results, Supplementary Figure S1 and Supplementary Table S3.

Insecticidal activity and presence of the Fit toxin

Functions encoded by the Fit gene cluster were demonstrated to contribute to insecticidal activity of the strains *P. protegens* strains CHA0 and Pf-5 and *P. chlororaphis* PCL1391 (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013; Kupferschmied *et al.*, 2014). Searching the genomes of the selected strains revealed that the gene cluster is present in all strains of sub-clade 1, but neither in sub-clade 2 nor sub-clade 3 (Figure 2), which is in line with results obtained by Ruffner *et al.* (2015).

All 26 strains were tested for their injectable and oral activity against insect larvae. A summary of the results is given in Figure 2. All strains of sub-clade 1 exhibited strong injectable and oral insecticidal activity whereas no strain of sub-clade 2 had an effect on larval survival in any of the test systems. However, the presence of the *fit* cluster, while indicative of strong insecticidal activity, does not seem to be the sole factor associated with the ability to kill insects, since also the tested strains of sub-clade 3, which do not contain the *fit* cluster caused some mortality, but to a much lower extent

than strains of sub-clade 1. Insecticidal activity was associated with specific phylogenetic subgroups, but did not correlate with the origin of the isolate (i.e., root or non-root habitat).

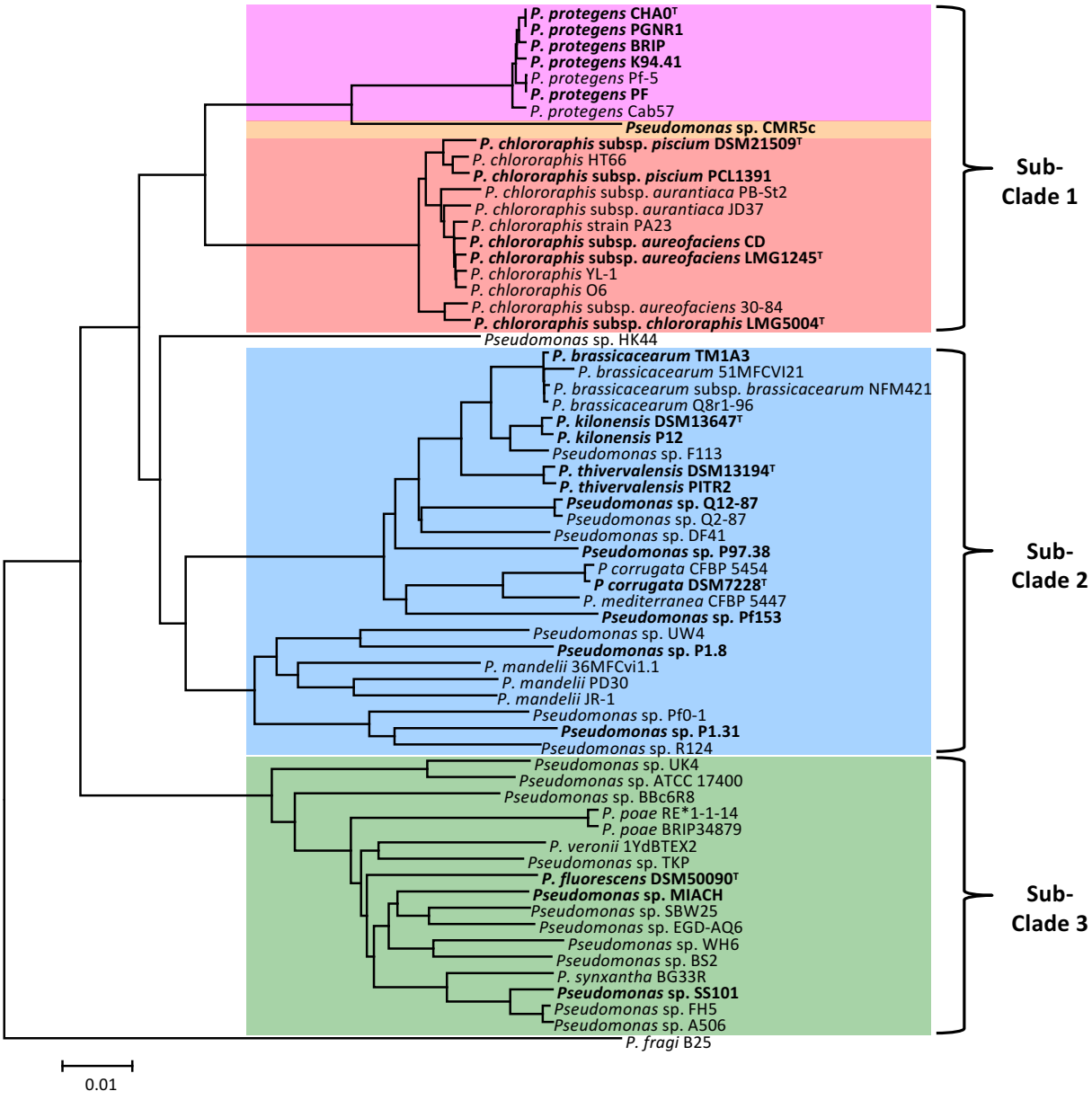


Figure 1 Phylogeny of the *P. fluorescens* group based on the core genome. Genomes sequenced in this study and high quality genomes that are publicly available by February 2015 were used to generate a core genome tree in EDGAR. Strains investigated in this study are depicted in bold. Sub-clades were defined after Loper *et al.* (2012). Sub-clade 1 corresponds to the *P. chlororaphis* subgroup, sub-clade 3 to the *P. fluorescens* subgroup, and sub-clade 2 comprises strains belonging to three different subgroups within the *P. fluorescens* group according to Mulet *et al.* (2012), see also Supplementary Figure S1.

To mimic a systemic infection, bacteria were injected into the hemocoel of *G. mellonella* larvae (Figure 3A). All Fit-producing strains, i.e., the entire sub-clade 1, were able to cause 100% mortality within the first 48 h, confirming and extending results of an earlier study demonstrating sub-clade 1 strains to cause 100% mortality when injected into *G. mellonella* (Ruffner *et al.*, 2015). Although all strains in sub-clade 1 are highly insecticidal, strain-specific differences for killing rate were observed. The two strains *Pseudomonas* sp. CMR5c and CMR12a, that probably represent a new species within sub-clade 1, were killing more rapidly than *P. protegens* and *P. chlororaphis* as indicated by significantly shorter times to reach 50% larval mortality (LT_{50}) (Table 2). *P. chlororaphis* subsp. *aureofaciens* strains CD and LMG 1245^T have higher LT_{50} values compared to strains of the *P. chlororaphis* subspecies *piscium* and *chlororaphis* (Table 2). Thus, the kill-time reflects phylogenetic relationships, which may be explained by the presumably multifactorial nature of insecticidal activity of fluorescent pseudomonads. Beside a common arsenal of contributing factors harbored by all insecticidal strains, some specific factors may exist, which enable certain strains or closely related groups of strains to kill more efficiently than others. No injectable activity was found for strains of sub-clade 2 (Figure 3A). In contrast, two strains of sub-clade 3, namely *P. fluorescens* DSM 50090^T and *Pseudomonas* sp. SS101, both lacking the *fit* genes, caused lethal infections in *G. mellonella*. However, mortality caused by these strains was delayed compared to infections with most strains of sub-clade 1 (Table 2) and larvae lack the strong melanization response and the floppy phenotype observed after infection by sub-clade 1 (Figure 3C). These symptoms might be attributed to the Fit toxin as larvae injected with a *fitD* deletion mutant of *P. protegens* CHA0 lack these phenotypes (Péchy-Tarr *et al.*, 2008) similarly to larvae injected with SS101 or DSM 50090^T. While injectable insecticidal activity seems to be universal to strains of sub-clade 1 this is not the case for sub-clade 3. The third tested strain of this sub-clade, *Pseudomonas* sp. MIACH, was not able to kill insect larvae upon injection (Figure 3A), although larvae started to slightly melanize at 1-day post infection (Figure 3D).

In natural infections, a bacterium first has to breach several barriers to reach the hemocoel. Therefore, the selected strains were further tested for oral activity against *P. xylostella* larvae. All strains that carry the *fit* genes were able to cause high mortality within three days (Figure 3B, Supplementary Table S4).

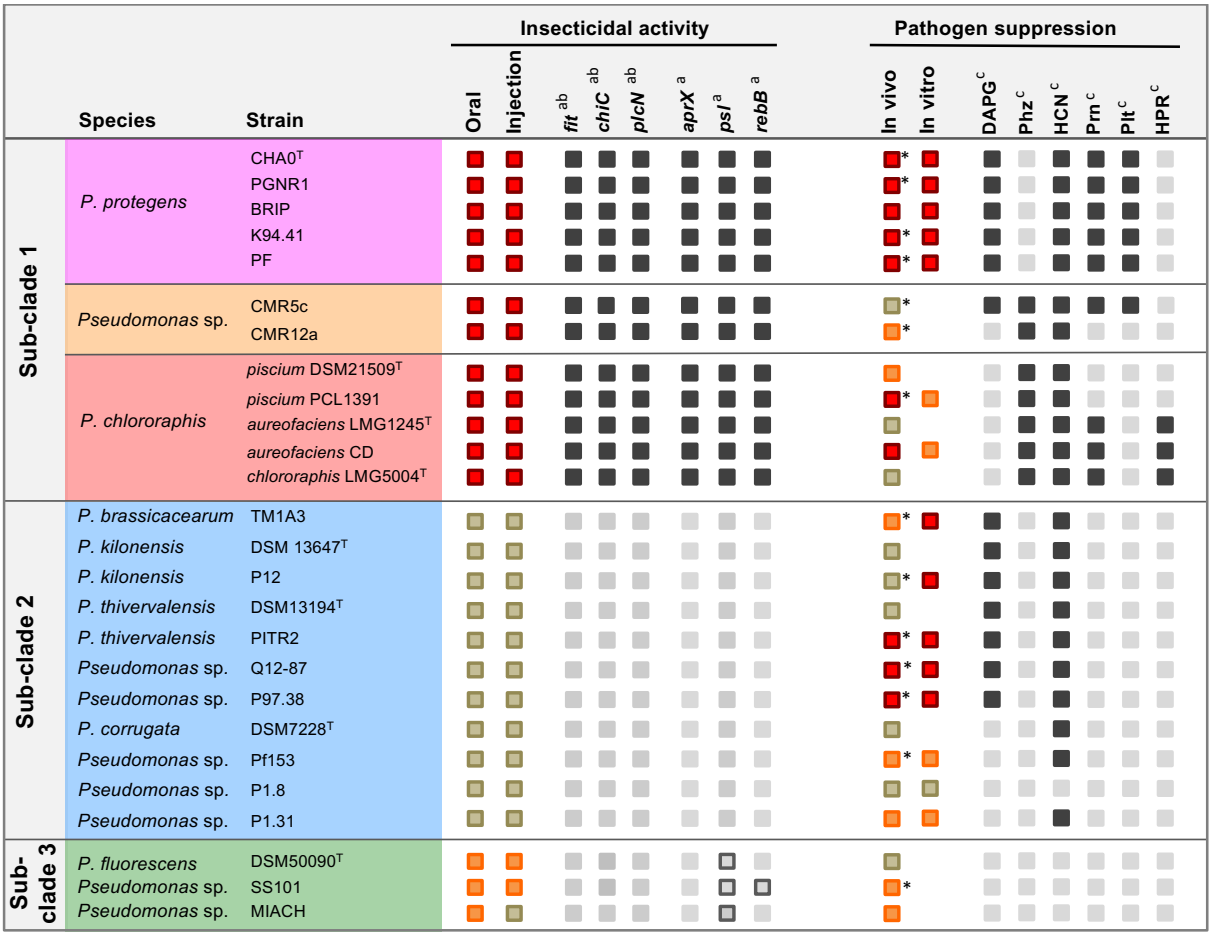
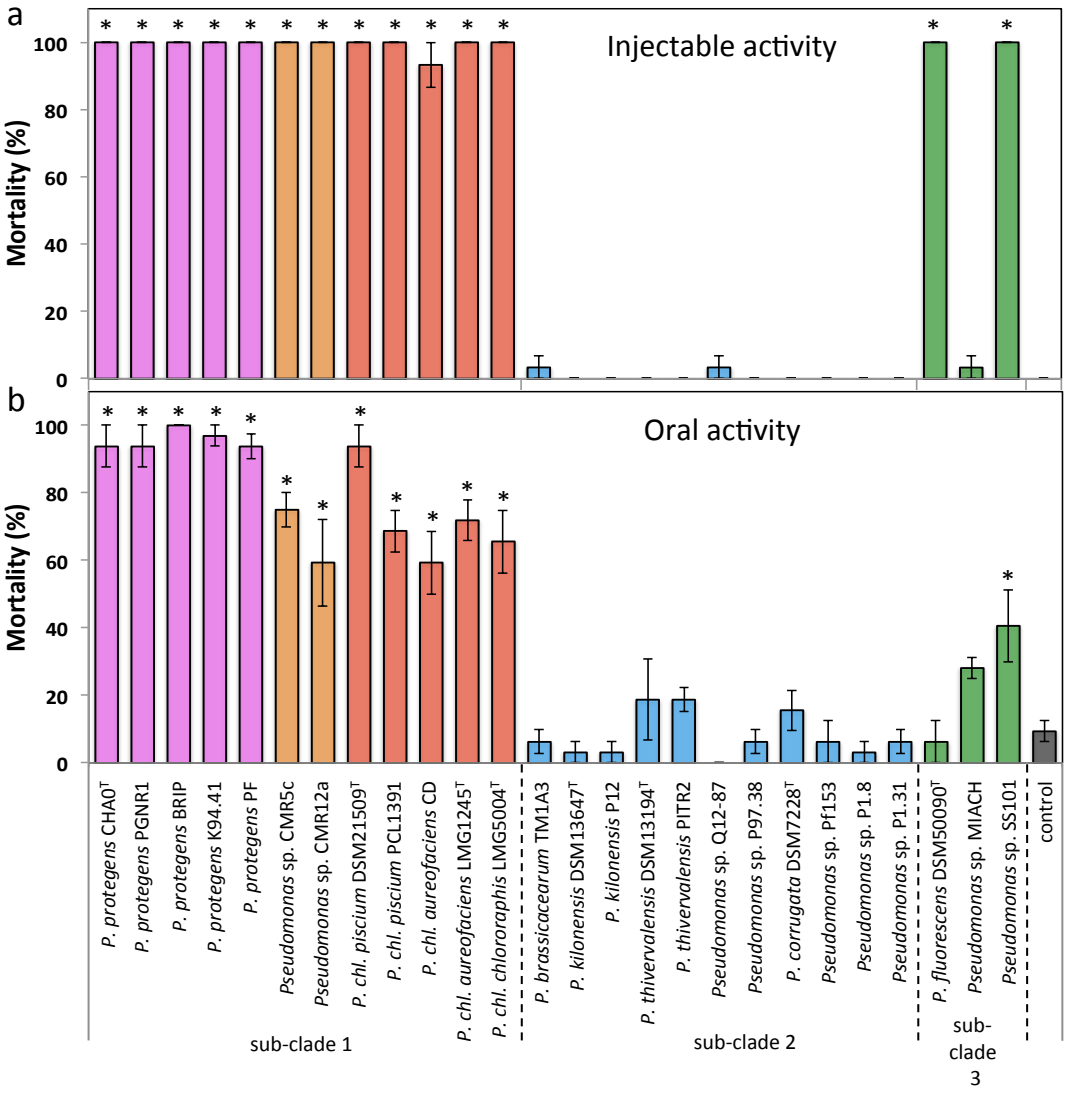
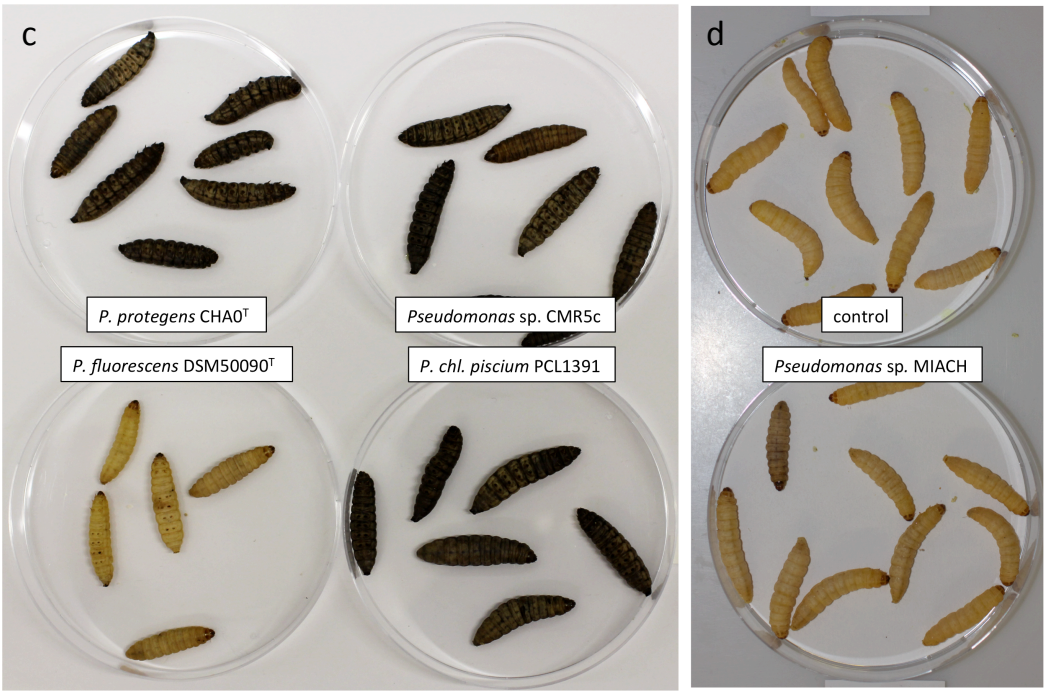


Figure 2 Overview on insecticidal activity, pathogen suppression and presence of associated gene clusters in 26 strains of the *P. fluorescens* group. Colored boxes represent activity against insects and plant pathogens as assessed within this study: high activity, medium activity, no activity. Insecticidal activity was assessed in injection assays against *G. mellonella* larvae and feeding assays against *P. xylostella* and *S. littoralis* larvae, and depicted activities are based on the results presented in Figure 3, Table 2, Supplementary Figure S2 and Supplementary Table S4. Disease suppression was assessed in a cucumber-*Pythium ultimum* assay and activities are based on the data depicted in Supplementary Table S5. Strains indicated by an asterisk were reported to have biocontrol activity against plant diseases in earlier studies (see Table 1). *In vitro* inhibition of mycelial growth was assessed on two media against *P. ultimum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* and activities are based on the results shown in Supplementary Figure S3. Grey boxes represent presence of selected genes/gene clusters that were found to be associated with insecticidal strains (this study) or that are required for the production of the indicated antifungal metabolites. present, partially present, absent. Exact loci, which were checked for presence/absence, are indicated in Supplementary Table S1. There, additional genes as well as all additional strains are presented. [a] Selected genes that were identified by comparative genomics to be specific for strains that show insecticidal activity. A complete list is presented in Supplementary Table S6. *P. fluorescens* insecticidal toxin-cluster (*fit*), chitinase C (*chiC*), phospholipase C (*plcN*), metallopeptidase AprX (*aprX*), *rebB*-cluster (*rebB*), *psl*-cluster (*psl*). [b] Genes that were shown to contribute to insecticidal activity in this study (*chiC* and *plcN*) or elsewhere (*fit*) (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013). [c] Presence/absence of gene clusters required for the production of the indicated antifungal metabolites. 2,4-diacetylphloroglucinol (DAPG), phenazine (Phz), hydrogen cyanide (HCN), pyrrolnitrin (Prn), pyoluteorin (Plt), 2-hexyl-5-propyl-alkylresorcinol (HPR).

In contrast to the injection assays, here *P. protegens* were the most efficient insect killers in terms of extent and pace (Table 2). None of the strains of sub-clade 2 caused higher mortality than observed for control larvae (Figure 3B). This result was confirmed in a second oral test system where a selection of 15 strains of sub-clades 1 and 2 was fed to *S. littoralis* larvae (Supplementary Methods). No sub-clade 2 strain was able to kill the larvae, whereas all Fit-producing strains showed strong insecticidal activity (Supplementary Figure S2A). Thus, sub-clade 2 strains lack crucial traits enabling them to kill lepidopteran larvae. However, the lack of killing potential does not necessarily mean that these strains might not be able to persist in the insect gut. Persistence without killing could be a clever strategy to use the insect as a means of dispersal as a living insect will transport the bacteria further than a dead one. Monitoring bacterial cells revealed that all strains of sub-clade 1 were able to multiply within the *S. littoralis* larvae (data only shown for CHA0, Supplementary Figure S2B) and to reach about 10^8 cfu per larva while large differences were observed for strains in sub-clade 2. Several strains, namely *Pseudomonas* sp. P97.38, Q12-87 and P1.31, were indeed able to persist at levels of 10^6 to 10^7 cfu per larva, whereas others such as *P. thivervalensis* PITR2, *P. kilonensis* P12 or *Pseudomonas* sp. P1.8 underwent a 1000-fold population decline within a few days (Supplementary Figure S2B) indicating that they were cleared from the gut. Thus, although not having the ability to kill insect larvae, some strains of sub-clade 2 seem to possess features allowing certain persistence in insects.

Right side: Figure 3 Oral and systemic insecticidal activity is restricted to strains of specific phylogenetic subgroups within the *Pseudomonas fluorescens* group. (A) Systemic activity against *Galleria mellonella*. Larvae were injected with 4 x 10^4 bacterial cells. Bars show average mortality of three replicates with 10 larvae after 48 h. The experiment was repeated twice and highly similar results were obtained. **(B)** Oral activity against *Plutella xylostella*. Larvae were exposed to artificial diet covered with 8 x 10^7 bacterial cells. Bars show average mortality of four replicates with eight larvae after three days. The experiment was repeated and similar results were obtained (Supplementary Table S4). Error bars show standard error of the mean. Asterisks indicate strains that were significantly different from control larvae treated with 0.9% NaCl based on multiple comparisons by Kruskal-Wallis adjusted by Bonferroni-Holm ($P \leq 0.05$). **(C)** Typical melanization symptoms observed after 32 h in infections with *P. protegens* CHA0^T, *P. chlororaphis* subsp. *piscium* PCL1391, *Pseudomonas* sp. CMR5c, but not with *P. fluorescens* DSM 50090^T. **(D)** Although larvae injected with *Pseudomonas* sp. MIACH do not die, they become slightly melanized compared to control larvae. *P. chl.* stands for *Pseudomonas chlororaphis.*


a
Injectable activity
Mortality (%)
b
Oral activity
Mortality (%)
P. protegens CHA0T
P. protegens PGNR1
P. protegens BRIP
P. protegens K94.41
P. protegens PF
Pseudomonas sp. CMR5c
Pseudomonas sp. CMR12a
P. chl. piscium DSM21509T
P. chl. piscium PCL1391
P. chl. aureofaciens CD
P. chl. aureofaciens LMG1245T
P. chl. chlororaphis LMG5004T
P. brassicacearum TM1A3
P. kilonensis DSM13647T
P. kilonensis P12
P. thivervalensis DSM13194T
P. thivervalensis PITR2
Pseudomonas sp. Q12-87
Pseudomonas sp. P97.38
P. corrugata DSM7228T
Pseudomonas sp. Pf153
Pseudomonas sp. P1.8
Pseudomonas sp. P1.31
P. fluorescens DSM50090T
Pseudomonas sp. MIACH
Pseudomonas sp. SS101
control
sub-clade 1
sub-clade 2
sub-clade 3


c
P. protegens CHA0T
Pseudomonas sp. CMR5c
P. fluorescens DSM50090T
P. chl. piscium PCL1391
d
control
Pseudomonas sp. MIACH

In contrast to sub-clade 2 strains, sub-clade 3 strains were found to cause lethal oral infections in *P. xylostella*, which is to our knowledge the first report for strains of this sub-clade to orally kill lepidopteran insect larvae. However, similar to the results of the injection assay, strains of sub-clade 3 appeared to have strongly reduced oral activity compared to strains of sub-clade 1. This is in line with observations of Olcott *et al.* (2010), who described that oral infections of *D. melanogaster* with *Pseudomonas* sp. SBW25 (sub-clade 3) were less detrimental than infections with *P. protegens* Pf-5 (sub-clade 1). Strain *Pseudomonas* sp. SS101 had significant oral insecticidal activity in all repetitions of the experiment (Figure 3B, Supplementary Table S4). However, killing occurred slower and to a lower extent than it was the case for infections with strains of sub-clade 1. *P. fluorescens* DSM 50090^{T} which had injectable activity against *G. mellonella* showed either no or weak insecticidal activity when fed to *P. xylostella*. We hypothesize that this strain faces difficulties to breach the gut barrier on its own, but can act as an opportunistic pathogen taking the chance when an insect gets injured or weakened by other factors. More puzzling, is the outcome for *Pseudomonas* sp. MIACH, which, in spite of not killing larvae in injection experiments, seems to have slight oral activity. Strain MIACH caused mortality rates of 30 - 53% though the effect was significant only in one of the two experiments (Figure 3B, Supplementary Table S4). We hypothesize that this strain is able to do some damage to the insect gut, without killing the insect itself, thereby promoting a secondary infection by other microbes which invade the hemocoel and lead to larval death. Another explanation would be that this strain is less of a generalist and causes lethal infections only in certain insect species. As we tested injectable and oral activity in different insect species, we cannot exclude this possibility. Although some strain-specific differences exist, we conclude that strains in sub-clade 3 mostly possess some insecticidal activity but that it is by far less distinct than in Fit producing strains of sub-clade 1. To date no factor contributing to pathogenicity of sub-clade 3 strains has been identified, but it was suggested that so-called toxin complexes (Tc), first discovered in the entomopathogen *Photorhabdus luminescens*, could play a role (Loper *et al.*, 2012). In accordance to the study of Loper *et al.* (2012) different Tc-related genes could be identified in the genomes of the strains included in this study, but they were not restricted to the strains with insecticidal activity (data not shown). Hence, they might play a rather subtle role in *Pseudomonas*-insect associations.

Table 2 Lethal time 50 (LT_{50}) values for *Galleria mellonella* and *Plutella xylostella* larvae upon injection or oral uptake, respectively, of *Pseudomonas* strains.

Sub-clade	Strain	Injection, LT_{50} (h)	Oral, LT_{50} (d)
Sub-clade 1	*P. protegens* CHA0^T	26.3 (25.9; 26.6)^{b}	1.65 (1.44; 1.86)^{a}
	P. protegens CHA0^T	26.9 (26.5; 27.4)$^{\dagger\,\gamma}$	
	P. protegens PGNR1	29.3 (28.9; 29.8)^{de}	1.74 (1.56; 1.92)^{ab}
	P. protegens BRIP	29.0 (28.6; 29.4)^{d}	1.58 (1.40; 1.76)^{a}
	P. protegens K94.41	26.3 (25.9; 26.7)^{b}	1.58 (1.40; 1.76)^{a}
	P. protegens PF	34.4 (33.3; 35.6)^{g}	1.63 (1.43; 1.83)^{a}
	Pseudomonas sp. CMR5c	24.5 (24.2; 24.9)$^{\dagger\,\beta}$	2.24 (1.98; 2.49)^{c}
	Pseudomonas sp. CMR12a	22.0 (21.5; 22.6)$^{\dagger\,\alpha}$	2.63 (2.31; 2.95)^{c}
	P. chl. piscium DSM 21509^T	27.2 (26.7; 27.6)^{c}	1.66 (1.46; 1.86)^{a}
	P. chl. piscium PCL1391	24.9 (24.5; 25.3)^{a}	2.19 (1.87; 2.51)^{bc}
	P. chl. aureofaciens LMG 1245^T	30.1 (29.6; 30.6)^{e}	2.56 (2.24; 2.89)^{c}
	P. chl. aureofaciens CD	33.7 (32.8; 34.7)^{g}	2.08 (1.76; 2.39)^{abc}
	P. chl. chlororaphis LMG 5004^T	26.7 (26.2; 27.1)^{bc}	2.33 (1.97; 2.68)^{c}
Sub-clade 2	*P. brassicacearum* TM1A3	NA	NA
	P. kilonensis DSM 13647^T	NA	NA
	P. kilonensis P12	NA	NA
	P. thivervalensis DSM 13194^T	NA	NA
	P. thivervalensis PITR2	NA	NA
	Pseudomonas sp. Q12-87	NA	NA
	Pseudomonas sp. P97.38	NA	NA
	P. corrugata DSM 7228^T	NA	NA
	Pseudomonas sp. Pf153	NA	NA
	Pseudomonas sp. P1.8	NA	NA
	Pseudomonas sp. P1.31	NA	NA
Sub-clade 3	*P. fluorescens* DSM 50090^T	32.0 (31.4; 32.7)†	NA
	Pseudomonas sp. MIACH	NA	NA
	Pseudomonas sp. SS101	37.9 (36.9; 38.8)$^{\dagger\,\delta}$	NA
control	0.9 % NaCl	NA	NA

Galleria mellonella larvae were injected with 4 x 10^4 washed bacterial cells of the indicated strains. *Plutella xylostella* larvae were exposed to food pellets inoculated with 8 x 10^7 bacterial cells. LT_{50} values are estimates based on the generalized linear model using the MASS package in R (Venables and Ripley, 2002). LT_{50} estimates were calculated from three replicates with ten larvae per replicate for *G. mellonella* and from four replicates with eight larvae per replicate for *P. xylostella*. Numbers in brackets depict 95% confidence intervals for LT_{50} and significantly different values within the same column are followed by different letters. † These strains were tested in a separate experiment. NA = no LT_{50} value was calculated, because end mortality was less than 50%. *P. chl.* = *Pseudomonas chlororaphis*

Plant-beneficial effects are phylogenetically less predictable than insecticidal activity

Although biocontrol activity against root pathogens has been demonstrated for many strains of the *P. fluorescens* group, most of the species type strains have never been investigated. The lack of knowledge for these strains and for the new strains from non-root habitats led us to test all 26 strains investigated for insecticidal activity also for their biocontrol activity against the oomycete pathogen *Pythium ultimum* on cucumber roots and a subset of strains also for their in vitro inhibition of *P. ultimum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Biocontrol activity appeared to be phylogenetically less predictable than insecticidal activity, as effective as well as poor biocontrol strains were found throughout all the three sub-clades (Supplementary Results, Figure 2, Supplementary Table S5, Supplementary Figure S3). Similar to the results on insecticidal activity, no connection between the original habitat and the degree of plant protection was observed. Together, the bioassays with insects and pathogens identified several strains of sub-clade 1, which exhibit potent dual activity against plant pests and diseases and therefore could be of interest for implementation in integrated crop protection strategies.

Comparative genomics to identify potential factors associated to insecticidal activity

Draft genomes of all selected strains were generated with exception of the strains *P. protegens* CHA0 and *Pseudomonas* sp. SS101, for which the genomes were already available (Loper *et al.*, 2012; Jousset *et al.*, 2014), and *Pseudomonas* sp. CMR12a, for which the genome description will be released elsewhere and which was therefore not included in the comparative genomics analysis. The average number of contigs per genome was 32 (Supplementary Table S6). The obtained genome sizes range between 6.06 and 7.07 Mbp, which is in accordance to genome sizes obtained for other fluorescent pseudomonads (Loper *et al.*, 2012).

The next step was to search for genes that are common and unique to insecticidal strains, encoding candidate factors potentially involved during the infection of insect larvae. Using EDGAR (Blom *et al.*, 2009), we identified 90 genes that are present in all highly insecticidal strains (sub-clade 1), but neither in moderately insecticidal strains (sub-clade 3) nor in non-insecticidal strains (sub-clade 2) (Table 3). We further

identified 117 genes that are present in all strains of sub-clade 1 as well as in one or several of the strains in sub-clade 3, but again in none of the strains of sub-clade 2 (Table 3). A full list of all identified genes can be found in Supplementary Table S7. It comprises about 28 putative transporters, 21 putative regulatory genes and over 100 enzymes and hypothetical proteins that are unique to insecticidal strains (Supplementary Table S7). Amongst the identified transporters, there are several putative amino acid transporters. Insects are very rich in amino acids (Rumpold and Schluter, 2013) and thus these transporters might help to exploit the insect as a source of nutrients. The Fit toxin is specifically expressed in insects but not on plant roots (Péchy-Tarr *et al.*, 2013). This could also be the case for other virulence factors and might involve some of the many regulatory genes that were found to be specific to insecticidal strains. However, besides the Fit toxin (Péchy-Tarr *et al.*, 2008; Péchy-Tarr *et al.*, 2013; Ruffner *et al.*, 2013), no other insecticidal toxin was identified. For most of the 207 genes unique to insecticidal strains, a prediction on the biological function of the encoded product as well as on a possible role during the infection of insects would be very speculative at the present stage. Nevertheless, the comparative genomics also revealed several genes encoding proteins with homology to known virulence factors of other bacteria and that are of interest in terms of a possible association with insecticidal activity. Presence of those genes, which are discussed below, is indicated for our selection of strains in Figure 2 and for all strains included in the phylogeny of Figure 1 in Supplementary Table S1.

Upon ingestion of pathogenic bacteria, insects produce reactive oxygen species, antimicrobial peptides (AMPs) and lysozymes to rapidly eliminate infesting bacteria (Lemaitre and Hoffmann, 2007). One mechanism to counter this first line of insect immunity is to produce enzymes degrading AMPs. Exoproteases such as the Zn-dependent metallo-peptidase AprX, also called serralysin and the AprA alkaline protease were suggested to play a role during the early phase of bacterial infections (Liehl *et al.*, 2006). The gene *aprA* is present in all 25 genomes, except that of strain P1.8, whereas *aprX* was only detected in the genomes of strains belonging to sub-clade 1 (Supplementary Table S1). AprA and AprX belong to the M10 family that includes serralysin, aeruginolysin and other related exopeptidases that cause tissue damage and anaphylactic responses (Park and Ming, 2002). In *Pseudomonas entomophila*, an *aprA* mutant was shown to be slightly less virulent and to have a reduced persistence in *D.*

melanogaster (Liehl *et al.*, 2006). Serralysin of *Serratia marcescens* was shown to promote hemolymph bleeding in the silkworm (*Bombyx mori*) (Ishii *et al.*, 2014).

If bacteria persist within the insect gut, living cells or their toxins must breach the peritrophic membrane, a gut-delimiting chitinous matrix, to access the hemocoel (Vallet-Gely *et al.*, 2008). Chitinases affecting the peritrophic matrix are therefore potential virulence factors of entomopathogenic bacteria; for instance, chitinases of *B. thuringiensis* subsp. *israelensis* IPS68 and *B. thuringiensis* subsp. *aizawai* HD133 were shown to contribute to insecticidal activity towards *Culicuoides nubeculosus* and *S. littoralis,* respectively (Sampson and Gooday, 1998). In insecticidal strains of the *P. fluorescens* group, we identified two chitinase genes. The chitinase gene *chiC* encoded next to a chitin-binding protein is present exclusively in genomes of sub-clade 1 strains, whereas the second chitinase is present in nearly all *P. chlororaphis* strains and some sub-clade 3 strains (Supplementary Table S1).

Table 3 Genes associated with insecticidal activity.

Sub-clade 1	Sub-clade 2	Sub-clade 3			Number of Genes
		SS101	DSM 50090^T	MIACH	
+	–	–	–	–	90
+	–	+	+	+	57
+	–	+	+	–	20
+	–	+	–	+	7
+	–	+	–	–	20
+	–	–	+	+	13
+	–	–	+	–	0
+	–	–	–	+	0

Numbers of genes that are specific to insecticidal strains. Presence of genes was defined as 70% amino acid pairwise identity over at least 70% of gene length for the pairwise comparisons. Only genes that were common to all strains of sub-clade 1 (highly-insecticidal strains), but not found in any strain of sub-clade 2 (non-insecticidal strains) were considered.

+ indicates genes present in all these strains

– indicates genes absent in all these strains

PCL1391_2966 encodes for a phosphocholine-specific phospholipase C. This gene, *plcN*, was detected only in sub-clade 1 strains. Phospholipases are recognized as major virulence determinants in a number of bacterial species, including human, animal and several invertebrate pathogens (Songer, 1997; Farn *et al.*, 2001; Yang *et al.*, 2012).

The *ymt* gene encoding for a phospholipase D in *Yersinia pestis*, for example, is needed for persistence in the flea midgut (Hinnebusch *et al.*, 2002). Phospholipase C produced by *Mycobacterium abscessus* is crucial for survival in amoeba and is suggested to cause damage to mouse macrophages presumably by hydrolysis of membrane phospholipids (N'goma *et al.*, 2015).

Three small genes with homology to *reb* genes were found to be present in all strains of sub-clade 1 and in *Pseudomonas* sp. SS101, the strain with the highest insecticidal activity of sub-clade 3. Such *reb* genes have been mainly studied in *Caedibacter taenospiralis,* a *Paramecium* endosymbiont. They encode R-bodies, highly insoluble protein ribbons that are typically coiled into cylindrical structures but can unroll under certain conditions (Pond *et al.*, 1989) and are associated with the killing trait towards sensitive *Paramecia* (Dilts and Quackenbush, 1986). Orthologs of *reb* were found to be present in many free-living bacteria, but their function remains unclear to date (Raymann *et al.*, 2013).

A whole cluster of genes specific to insecticidal strains (loci PCL1391_4983 to PCL1391_4994) has high percentage of sequence identity to the *psl* gene cluster of *P. aeruginosa* which specifies the production of the extracellular polysaccharide Psl (Franklin *et al.*, 2011). Psl was shown contributing to biofilm production, tolerance to oxidizing agents and host defensive processes (Friedman and Kolter, 2004; Jackson *et al.*, 2004; Mishra *et al.*, 2012), i.e. traits likely useful in insect interactions.

Other factors, which still have to be kept in mind, are the antimicrobial compounds, such as 2,4-diacetylphloroglucinol, phenazine, pyoluteorin, pyrrolnitrin and hydrogen cyanide which are crucial for biocontrol activity against fungal diseases, although none is shared by all or unique to insecticidal strains (Figure 2, Supplementary Table S1) (Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Lugtenberg and Kamilova, 2009). However, some have activity against a broad spectrum of organisms including plants, nematodes, arthropods and even mammalian cells (Maurhofer *et al.*, 1992; Devi and Kothamasi, 2009; Kwak *et al.*, 2011; Neidig *et al.*, 2011; Nisr *et al.*, 2011; Jang *et al.*, 2013) and thus could contribute to *Pseudomonas*-derived insecticidal activity.

Chitinase ChiC and phospholipase PlcN contribute to oral insecticidal activity

In order to verify that our combination of bioassays and comparative genomics indeed led to the identification of valuable candidate genes associated with bacterial virulence towards insects, we generated, in model strain *P. protegens* CHA0, in-frame deletion mutants for selected genes: *plcN*, *chiC*, *aprX* and the cluster encoding homologs of *rebB*. None of the mutants differed in activity from the wild type when injected directly into the hemocoel of *G. mellonella* (Figure 4A, Supplementary Figure S4A and C). However, the *chiC* mutant was always significantly delayed in killing *P. xylostella* larvae upon ingestion (Figure 4B, Supplementary Figure S5A). We therefore conclude that the chitinase C, encoded by *chiC*, a gene common and unique to highly insecticidal strains, contributes to oral insect pathogenicity of *P. protegens* CHA0. Chitinase C was found to be responsible for chitobiosidase as well as endochitinase activity of *P. protegens* CHA0 as the *chiC* mutant exhibited no residual chitinase activity (Figure 4C). Since also a *gacA* mutant completely lost chitinase activity (Figure 4C) we believe that we identified with the *chiC* one of the hitherto unknown Gac-regulated virulence factors involved in oral insecticidal activity. Furthermore, the mutant deficient for phopholipase C (*plcN)* also showed reduced oral activity against *P. xylostella*. Although less distinct than for the *chiC* mutant, a delay in killing was always observed for the *plcN* mutant but the effect was not significant in all experiments (Supplementary Figures S4B and S5B).

In contrast, no difference to the wild type strain CHA0 was found for the *rebB1-3* and *aprX* mutants (Supplementary Figure S4D). However, these results do not exclude a role of factors, encoded by these genes, under different conditions or in an interaction with other insect species. Accordingly, the impact of the well–characterized Fit toxin on virulence towards insects also varies between insect species. Thus, a *fitD* mutant compared to the wild type *P. protegens* CHA0 is more strongly reduced in virulence towards *S. littoralis* than towards *P. xylostella* (data not shown).

The mutational analysis performed in this study gives only a first insight into the possible contribution of interesting candidate genes identified in the comparative genomics approach to insecticidal activity. An in-depth analysis of the role of chitinase C and phospholipase C would include the complementation of these mutants and will be subject to further studies.

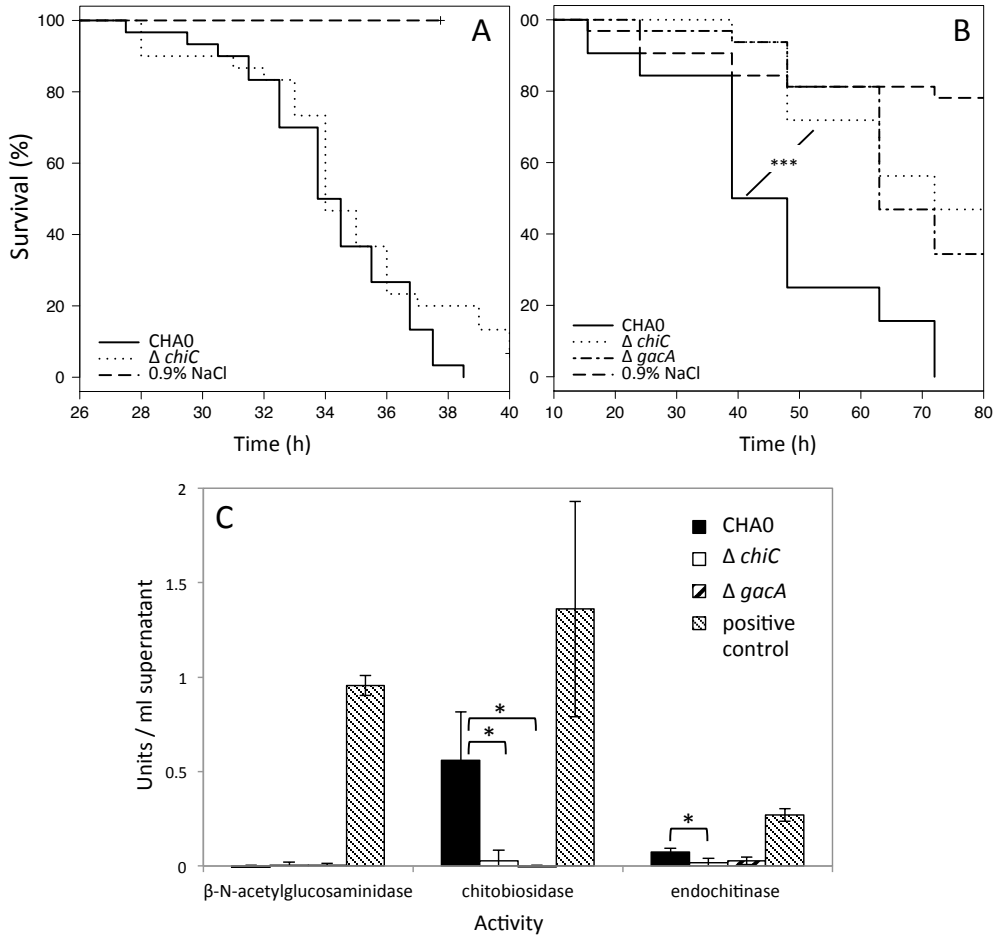


Figure 4 A derivative of *P. protegens* CHA0 deficient for a specific chitinase is reduced in oral, but not in injectable activity against insect larvae. (A) Systemic activity against *Galleria mellonella*. 30 larvae per treatment were injected with 2 x 10^3 bacterial cells and survival was recorded hourly. **(B)** Oral activity against *Plutella xylostella*. Larvae were exposed to artificial diet inoculated with 4 x 10^6 bacterial cells. Significant differences according to a Log-Rank test (Survival Package in R) between treatments with the wild type CHA0 and the chitinase C- negative mutant (Δ*chiC*) are indicated with ***(*P* <0.0001). Each mutant was tested at least three times with similar results. A repetition of the feeding assay is depicted in Supplementary Figure S5. **(C)** Chitinase activity of wild type CHA0 and its *chiC* mutant was assessed using a chitinase assay kit (Sigma, St. Louis, MO, USA). Three different substrates were used to test for exo- (β-N-acetylglucosaminidase and chitobiosidase) and endochitinase activity. Treatments indicated by an asterisk are significantly different based on a t-test ($p \leq 0.05$). CHA0, wild type; Δ*chiC*, chitinase C-negative mutant; Δ*gacA*, GacA-negative mutant; 0.9% NaCl served as negative control in the virulence assay; a positive control for chitinase activity was provided by the chitinase assay kit.

CONCLUSIONS

We provide the first extensive overview on insecticidal activity in the *P. fluorescens* group. Whilst biocontrol activity against fungal pathogens occurs throughout all studied sub-clades, insecticidal activity is unique to sub-clades 1 and 3. Only strains of sub-clade 1 display strong oral insecticidal activity and only they produce the Fit toxin. Intriguingly, Fit seems to contribute to the floppy and melanized phenotype associated with infections by highly pathogenic strains, however, the toxin is clearly not the major killing factor upon oral ingestion. Mutants of strains CHA0 and PCL1391 lacking the *fit* genes cause delayed, but still substantial mortality in *S. littoralis* (Ruffner *et al.*, 2013) when acquired via the oral infection route. By comparative genomics we now identified several candidate genes that might contribute to insecticidal activity and we demonstrated that the absence of two of these genes, encoding a specific chitinase and a phospholipase, negatively affects insecticidal activity. We hypothesize that especially the chitinase C might be involved during the gut stage of the infection process, causing damage to the peritrophic membrane. However, to understand the exact mode of action of these pathogenicity factors during the infection process, further investigations are needed. Nevertheless, the presented data highly increases the knowledge on the genetic basis of insecticidal activity of fluorescent pseudomonads and points to a multifactorial nature of this trait.

Although we provide evidence that many strains of the *P. fluorescens* group can be insect pathogenic and others might persist in insects as commensals, the ecological relevance of insects as a host for these bacteria is still elusive and an intriguing field for future research. The fact that certain pseudomonads, to date considered to be plant-associated, perform very well in a completely different habitat such as an insect raises the question whether these bacteria are indeed mainly plant-associated. Insecticidal as well as biocontrol activity against plant diseases was found to be independent of the original habitat of a strain. For example, closely related strains can be isolated from fish or cyclops and behave similarly well on roots as root isolates. This observation is in line with other studies that found the isolation source of a bacterial strain not to be predictive for its performance in another habitat (Alonso *et al.*, 1999; Grosso-Becerra *et al.*, 2014). For instance, Hilker and colleagues found no correlation between original habitat and virulence in different test systems for clinical and environmental clones of *P. aeruginosa* (Hilker *et al.*, 2015). In general, fluorescent pseudomonads might be quite

ubiquitous and probably possess an arsenal of traits allowing them to easily switch niches and to conquer the habitat they encounter. Insects could be especially useful as a means of dispersal, a phenomenon documented for diverse plant-pathogenic bacteria (Nadarasah and Stavrinides, 2011). Some *Pseudomonas syringae* strains for instance can use the pea aphid as alternative primary host where they replicate to high numbers and can be deposited onto a new plant host via excreted honeydew (Stavrinides *et al.*, 2009). Similarly, the rhizobacterium *P. chlororaphis* strain L11 was found to be transmittable from one plant to another by *Diabrotica undecimpunctata* subsp. *howardi* feeding on colonized plants (Snyder *et al.*, 1998). In contrast to considering the insect as an alternative host, one could even speculate that the plant is not the primary host for species like *P. protegens* and *P. chlororaphis,* but rather a transient host on which they endure until they encounter the next insect host. As research to date is very much biased towards plant-association, future studies especially on strains actually isolated from insects will be required to gain a better understanding of the importance insects have as hosts for strains of the *P. fluorescens* group.

Besides its ecological relevance, insecticidal activity might be of great agronomical interest. Our bioassays revealed several strains, especially of the species *P. protegens*, that display potent dual activity, killing insect larvae and protecting plants against pathogens. Fluorescent pseudomonads are already commercially used for the biological control of plant diseases (Stockwell and Stack, 2007; Berg, 2009). Our discovery of strains with the capacity to control insect pests on top of fungal pathogens renders these bacteria highly interesting for a new field of application and an additional market.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS AND CONTRIBUTORS

PF, BR, TS, CK and MM designed the research; TS, CK and MM supervised the study; PF and TS performed the genome sequencing, assembly and comparative genomics; AG and JB provided the infrastructure for the comparative genomics analysis and JB subjected the genomes to automatic annotation; TS and PF performed the phylogenetic analyses; GB, JF, MH and JR provided strains and MH, in addition, provided the genomic data on strains *Pseudomonas* sp. CMR5c and CMR12a; PF and MP generated the mutants; PF, NA, BR, SF and ZM performed the bioassays; AD and PF performed the chitinase activity assay; PF analysed the data; PF, BR, TS, CK and MM wrote the manuscript. All authors critically revised the manuscript and approved the final version.

FUNDING

This study was financed by grants obtained from the Swiss National Foundation for Scientific Research SNSF (Projects 31003A-138248, 31003A-120121A0 and 31003A-159520). Funding for BD and THMS was provided by the SNSF NRP 68 (Project 406840-143144). Syngenta Crop Protection (Stein, Switzerland) generously provided *Spodoptera littoralis* and *Plutella xylostella* larvae.

ACKNOWLEDGEMENTS

We thank Peter Kupferschmied for his help with the development of mutational strategies and the annotation of the genome of *P. chlororaphis* PCL1391. We acknowledge Sandra Siegfried, Nicolas Hofer, Tania Meylan, Dominique Fuchs, Michael Schläfli, Benjamin Laubi, Simona Crivelli and Christelle Chanez for technical assistance. We acknowledge technical assistance by the Bioinformatics Core Facility / Professorship of Systems Biology at JLU Giessen and access to resources financially supported by the BMBF grant FKZ 031A533 within the de.NBI network.

REFERENCES

Achouak W., Sutra L., Heulin T., Meyer J. M., Fromin N., Degraeve S. *et al.* (2000). *Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov., two root-associated bacteria isolated from *Brassica napus* and *Arabidopsis thaliana*. *Int J Syst Evol Microbiol* 50**:** 9-18.

Alonso A., Rojo F., Martinez J. L. (1999). Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin. *Environ Microbiol* 1**:** 421-430.

Bakker P. A. H. M., Pieterse C. M. J., van Loon L. C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology* 97**:** 239-243.

Berg G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 84**:** 11-18.

Bertani G. (1951). Studies on lysogenesis.1. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 62**:** 293-300.

Blom J., Albaum S. P., Doppmeier D., Puehler A., Vorhoelter F.-J., Zakrzewski M. *et al.* (2009). EDGAR: A software framework for the comparative analysis of prokaryotic genomes. *BMC Bioinformatics* 10**:** 154.

Burr S. E., Gobeli S., Kuhnert P., Goldschmidt-Clermont E., Frey J. (2010). *Pseudomonas chlororaphis* subsp. *piscium* subsp. nov., isolated from freshwater fish. *Int J Syst Evol Microbiol* 60**:** 2753-2757.

Chin-A-Woeng T. F. C., Bloemberg G. V., van der Bij A. J., van der Drift K., Schripsema J., Kroon B. *et al.* (1998). Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant-Microbe Interact* 11**:** 1069-1077.

D'aes J., Hua G. K., De Maeyer K., Pannecoucque J., Forrez I., Ongena M. *et al.* (2011). Biological control of *Rhizoctonia* root rot on bean by phenazine- and cyclic lipopeptide-producing *Pseudomonas* CMR12a. *Phytopathology* 101**:** 996-1004.

de Souza J. T., de Boer M., de Waard P., van Beek T. A., Raaijmakers J. M. (2003). Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. *Appl Environ Microbiol* 69**:** 7161-7172.

Devi K. K., Kothamasi D. (2009). *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting cytochromecoxidase of the termite respiratory chain. *FEMS Microbiol Lett* 300**:** 195-200.

Dilts J. A., Quackenbush R. L. (1986). A mutation in the R-body coding sequence destroys expression of the killer trait in *P. tetraurelia*. *Science* 232**:** 641-643.

Farn J. L., Strugnell R. A., Hoyne P. A., Michalski W. P., Tennent J. M. (2001). Molecular characterization of a secreted enzyme with phospholipase B activity from *Moraxella bovis*. *J Bacteriol* 183**:** 6717-6720.

Franklin M. J., Nivens D. E., Weadge J. T., Howell P. L. (2011). Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Front Microbiol* 2**:** 167.

Friedman L., Kolter R. (2004). Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J Bacteriol* 186**:** 4457-4465.

Fuchs J., Defago G. (1991). Protection of cucumber plants against black root rot caused by *Phomopsis sclerotioides* with rhizobacteria. *Bulletin SROP* 14**:** 57-62.

Fuchs J. G., Moenne-Loccoz Y., Defago G. (2000). The laboratory medium used to grow

biocontrol *Pseudomonas* sp. Pf153 influences its subsequent ability to protect cucumber from black root rot. *Soil Biol Biochem* 32**:** 421-424.

Gomila M., Peña A., Mulet M., Lalucat J., García-Valdés E. (2015). Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 6**:** 214.

Grosso-Becerra M. V., Santos-Medellin C., Gonzalez-Valdez A., Mendez J. L., Delgado G., Morales-Espinosa R. *et al.* (2014). *Pseudomonas aeruginosa* clinical and environmental isolates constitute a single population with high phenotypic diversity. *BMC Genomics* 15**:** 318

Gupta G. P., Rani S., Birah A., Raghuraman M. (2005). Improved artificial diet for mass rearing of the tobacco caterpillar, *Spodoptera litura* (Lepidoptera: Noctuidae). *Int J Trop Insect Sci* 25**:** 55-58.

Haas D., Keel C. (2003). Regulation of antibiotic production in root-colonizing *Peudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol* 41**:** 117-153.

Haas D., Défago G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 3**:** 307-319.

Herren J., Lemaitre B. (2012). Insect-microbe interactions: the good, the bad and the others. *Curr Opin Microbiol* 15**:** 217-219.

Hilker R., Munder A., Klockgether J., Losada P. M., Chouvarine P., Cramer N. *et al.* (2015). Interclonal gradient of virulence in the *Pseudomonas aeruginosa* pangenome from disease and environment. *Environ Microbiol* 17**:** 29-46.

Hinnebusch B. J., Rudolph A. E., Cherepanov P., Dixon J. E., Schwan T. G., Forsberg A. (2002). Role of Yersinia murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* 296**:** 733-735.

Ishii K., Adachi T., Hara T., Hamamoto H., Sekimizu K. (2014). Identification of a *Serratia marcescens* virulence factor that promotes hemolymph bleeding in the silkworm, *Bombyx mori*. *J Invertebr Pathol* 117**:** 61-67.

Jackson K. D., Starkey M., Kremer S., Parsek M. R., Wozniak D. J. (2004). Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J Bacteriol* 186**:** 4466-4475.

Jang J. Y., Yang S. Y., Kim Y. C., Lee C. W., Park M. S., Kim J. C. *et al.* (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J Agric Food Chem* 61**:** 6786-6791.

Jousset A., Schuldes J., Keel C., Maurhofer M., Daniel R., Scheu S. *et al.* (2014). Full-genome sequence of the plant growth-promoting bacterium *Pseudomonas protegens* CHA0. *Genome Announc* 2**:** e00322-00314.

Keel C., Weller D. M., Natsch A., Défago G., Cook R. J., Thomashow L. S. (1996). Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl Environ Microbiol* 62**:** 552-563.

Kluyver A. J. (1956). *Pseudomonas aureofaciens* nov. spec. and its pigments. *J Bacteriol* 72**:** 406-411.

Kruijt M., Tran H., Raaijmakers J. M. (2009). Functional, genetic and chemical characterization of biosurfactants produced by plant growth-promoting *Pseudomonas putida* 267. *J Appl Microbiol* 107**:** 546-556.

Kupferschmied P., Maurhofer M., Keel C. (2013). Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Frontiers in plant science* 4**:** 287.

Kupferschmied P., Péchy-Tarr M., Imperiali N., Maurhofer M., Keel C. (2014). Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial

Pseudomonas protegens. *PLoS Path* 10**:** e1003964.

Kwak Y.-S., Han S., Thomashow L. S., Rice J. T., Paulitz T. C., Kim D. *et al.* (2011). *Saccharomyces cerevisiae* genome-wide mutant screen for sensitivity to 2,4-diacetylphloroglucinol, an antibiotic produced by *Pseudomonas fluorescens*. *Appl Environ Microbiol* 77**:** 1770-1776.

Lemaitre B., Hoffmann J. (2007). The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 25**:** 697-743.

Levy E., Gough F. J., Berlin K. D., Guiana P. W., Smith J. T. (1992). Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescens* and its antibiotics. *Plant Pathol* 41**:** 335-341.

Liehl P., Blight M., Vodovar N., Boccard F., Lemaitre B. (2006). Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. *PLoS Path* 2**:** e56.

Loper J. E., Hassan K. A., Mavrodi D. V., Davis E. W., 2nd, Lim C. K., Shaffer B. T. *et al.* (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet* 8**:** e1002784.

Lugtenberg B., Kamilova F. (2009). Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* 63**:** 541-556.

Martinez-Garcia E., de Lorenzo V. (2011). Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Environ Microbiol* 13**:** 2702-2716.

Maurhofer M., Keel C., Schnider U., Voisard C., Haas D., Defago G. (1992). Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology* 82**:** 190-195.

Mazzola M., Zhao X., Cohen M. F., Raaijmakers J. M. (2007). Cyclic lipopeptide surfactant production by *Pseudomonas fluorescens* SS101 is not required for suppression of complex *Pythium* spp. populations. *Phytopathology* 97**:** 1348-1355.

Mercado-Blanco J., Bakker P. A. H. M. (2007). Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Anton Leeuw Int J G* 92**:** 367-389.

Meyer F., Goesmann A., McHardy A. C., Bartels D., Bekel T., Clausen J. *et al.* (2003). GenDB - an open source genome annotation system for prokaryote genomes. *Nucleic Acids Res* 31**:** 2187-2195.

Meyer J. B., Frapolli M., Keel C., Maurhofer M. (2011). Pyrroloquinoline quinone biosynthesis gene *pqqC*, a novel molecular marker for studying the phylogeny and diversity of phosphate-solubilizing pseudomonads. *Appl Environ Microbiol* 77**:** 7345-7354.

Mishra M., Byrd M. S., Sergeant S., Azad A. K., Parsek M. R., McPhail L. *et al.* (2012). *Pseudomonas aeruginosa* Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell Microbiol* 14**:** 95-106.

Mulet M., Gomila M., Scotta C., Sanchez D., Lalucat J., Garcia-Valdes E. (2012). Concordance between whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry and multilocus sequence analysis approaches in species discrimination within the genus *Pseudomonas*. *Syst Appl Microbiol* 35**:** 455-464.

N'goma J. C. B., Le Moigne V., Soismier N., Laencina L., Le Chevalier F., Roux A. L. *et al.* (2015). *Mycobacterium abscessus* phospholipase C expression is induced during coculture within Amoebae and enhances *M. abscessus* virulence in mice. *Infect Immun* 83**:** 780-791.

Nadarasah G., Stavrinides J. (2011). Insects as alternative hosts for phytopathogenic bacteria.

FEMS Microbiol Rev 35**:** 555-575.

Neidig N., Paul R. J., Scheu S., Jousset A. (2011). Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. *Microb Ecol* 61**:** 853-859.

Nisr R. B., Russell M. A., Chrachri A., Moody A. J., Gilpin M. L. (2011). Effects of the microbial secondary metabolites pyrrolnitrin, phenazine and patulin on INS-1 rat pancreatic beta-cells. *FEMS Immunol Med Microbiol* 63**:** 217-227.

Olcott M. H., Henkels M. D., Rosen K. L., Walker F. L., Sneh B., Loper J. E. *et al.* (2010). Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. *PloS one* 5**:** e12504.

Opota O., Vallet-Gely I., Vincentelli R., Kellenberger C., Iacovache I., Gonzalez M. R. *et al.* (2011). Monalysin, a novel -pore-forming toxin from the *Drosophila* pathogen *Pseudomonas entomophila*, contributes to host intestinal damage and lethality. *PLoS Path* 7**:** e1002259.

Park H. I., Ming L. J. (2002). Mechanistic studies of the astacin-like *Serratia* metalloendopeptidase serralysin: highly active (>2000%) Co(II) and Cu(II) derivatives for further corroboration of a "metallotriad" mechanism. *J Biol Inorg Chem* 7**:** 600-610.

Paulsen I. T., Press C. M., Ravel J., Kobayashi D. Y., Myers G. S., Mavrodi D. V. *et al.* (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol* 23**:** 873-878.

Péchy-Tarr M., Bruck D. J., Maurhofer M., Fischer E., Vogne C., Henkels M. D. *et al.* (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ Microbiol* 10**:** 2368-2386.

Péchy-Tarr M., Borel N., Kupferschmied P., Turner V., Binggeli O., Radovanovic D. *et al.* (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ Microbiol* 15**:** 736-750.

Peix A., Valverde A., Rivas R., Igual J. M., Ramirez-Bahena M. H., Mateos P. F. *et al.* (2007). Reclassification of *Pseudomonas aurantiaca* as a synonym of *Pseudomonas chlororaphis* and proposal of three subspecies, *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. *Int J Syst Evol Microbiol* 57**:** 1286-1290.

Perneel M., Heyrman J., Adiobo A., De Maeyer K., Raaijmakers J. M., De Vos P. *et al.* (2007). Characterization of CMR5c and CMR12a, novel fluorescent *Pseudomonas* strains from the cocoyam rhizosphere with biocontrol activity. *J Appl Microbiol* 103**:** 1007-1020.

Pond F. R., Gibson I., Lalucat J., Quackenbush R. L. (1989). R-body-producing bacteria. *Microbiol Rev* 53**:** 25-67.

Raaijmakers J. M., De Bruijn I., Nybroe O., Ongena M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol Rev* 34**:** 1037-1062.

Ramette A., Frapolli M., Fischer-Le Saux M., Gruffaz C., Meyer J.-M., Défago G. *et al.* (2011). *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. *Syst Appl Microbiol* 34**:** 180-188.

Raymann K., Bobay L.-M., Doak T. G., Lynch M., Gribaldo S. (2013). A genomic survey of Reb homologs suggests widespread occurrence of R-bodies in proteobacteria. *G3-Genes Genomes Genetics* 3**:** 505-516.

Rhodes M. E. (1959). The characterization of *Pseudomonas fluorescens*. *J Gen Microbiol* 21**:** 221-263.

Ruffner B., Péchy-Tarr M., Ryffel F., Hoegger P., Obrist C., Rindlisbacher A. *et al.* (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ Microbiol* 15**:** 751-763.

Ruffner B., Pechy-Tarr M., Hofte M., Bloemberg G., Grunder J., Keel C. *et al.* (2015). Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* 16**:** 609.

Rumpold B. A., Schluter O. K. (2013). Nutritional composition and safety aspects of edible insects. *Mol Nutr Food Res* 57**:** 802-823.

Sampson M. N., Gooday G. W. (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiol-UK* 144**:** 2189-2194.

Scarlett C. M., Fletcher J. T., Roberts P., Lelliott R. A. (1978). Tomato pith necrosis caused by *Pseudomonas corrugata* n. sp. *Ann Appl Biol* 88**:** 105-114.

Sikorski J., Stackebrandt E., Wackernagel W. (2001). *Pseudomonas kilonensis* sp. nov., a bacterium isolated from agricultural soil. *Int J Syst Evol Microbiol* 51**:** 1549-1555.

Smits T. H. M., Rezzonico F., Kamber T., Blom J., Goesmann A., Frey J. E. *et al.* (2010). Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. *Mol Plant-Microbe Interact* 23**:** 384-393.

Snyder W. E., Tonkyn D. W., Kluepfel D. A. (1998). Insect-mediated dispersal of the rhizobacterium *Pseudomonas chlororaphis*. *Phytopathology* 88**:** 1248-1254.

Songer J. G. (1997). Bacterial phospholipases and their role in virulence. *Trends Microbiol* 5**:** 156-161.

Stavrinides J., McCloskey J. K., Ochman H. (2009). Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. *Appl Environ Microbiol* 75**:** 2230-2235.

Stockwell V. O., Stack J. P. (2007). Using *Pseudomonas* spp. for integrated biological control. *Phytopathology* 97**:** 244-249.

Stutz E. W., Défago G., Kern H. (1986). Naturally-occuring fluorescent pseudomonads involved in suppression of black root-rot of tobacco. *Phytopathology* 76**:** 181-185.

Tran H., Ficke A., Asiimwe T., Hofte M., Raaijmakers J. M. (2007). Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. *New Phytol* 175**:** 731-742.

Vallet-Gely I., Lemaitre B., Boccard F. (2008). Bacterial strategies to overcome insect defences. *Nat Rev Microbiol* 6**:** 302-313.

Venables W. N., Ripley B. D. (2002). *Modern Applied Statistics with S*, Fourth edn. Springer: New York.

Wang C. X., Ramette A., Punjasamarnwong P., Zala M., Natsch A., Moenne-Loccoz Y. *et al.* (2001). Cosmopolitan distribution of *phlD*-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin. *FEMS Microbiol Ecol* 37**:** 105-116.

Yang G. W., Hernandez-Rodriguez C. S., Beeton M. L., Wilkinson P., Ffrench-Constant R. H., Waterfield N. R. (2012). Pdl1 is a putative lipase that enhances *Photorhabdus* toxin complex secretion. *PLoS Path* 8**:** e1002692.

SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Supplementary Results

Supplementary Figures

Supplementary Tables

References

Supplementary Materials and Methods

Sequencing of strain *Pseudomonas* sp. CMR5c

Paired-end sequence reads of genomic DNA of *Pseudomonas* CMR5c were generated using the Illumina HiSeq2500 system. The *de novo* assembly analysis was performed using the "*de novo* assembly" option of the CLC Genomics Workbench version 7.0.4. The scaffolding analysis was performed using the SSPACE Premium scaffolder version 2.3 (Boetzer *et al.*, 2011). Automated gap closure analysis was done using GapFiller version 1.10 (Boetzer and Pirovano, 2012). No further manual assembly was performed.

Bioinformatics

Housekeeping genes of sequenced strains were collected from the annotated genomes, cropped to the size of the fragments used for phylogeny and concatenated according to Mulet *et al.* (2012). Alignments were done using Muscle in MEGA v6.0, and a phylogenetic analysis was done with the maximum likelihood method.

Pairwise average amino acid identities (AAI) were calculated in EDGAR (Blom *et al.*, 2009). GGDC values are calculated using the Genome-to-Genome Distance Calculator Version 2 and reported according to formula 2, best suited when including draft genomes (Auch *et al.*, 2010; Meier-Kolthoff *et al.*, 2013).

Injection assay with *Galleria mellonella*

Washed bacterial cells (10 µl) suspended in 0.9% sterile NaCl solution and adjusted to the desired concentration were injected into the hemolymph of ultimate-instar *Galleria mellonella* larvae (Hebeisen Fishing, Zürich, Switzerland) using a 1-ml syringe with a 27-gauge needle in a repetitive dispensing Tridak Stepper (Intertronic, Oxfordshire, UK). Sterile 0.9% NaCl solution served as control. Three times ten larvae were injected per treatment and kept in Petri dishes at 24°C in the dark. Larvae were scored as live or dead regularly over two days. Mortality was defined as the inability of larvae to react to poking.

Feeding assay with *Spodoptera littoralis*

Food pellet assays with *Spodoptera littoralis* were performed as described by Ruffner et al. (2013). Briefly, third instar larvae of *S. littoralis* (Syngenta Crop Protection AG, Stein) were exposed to modified insect diet (Gupta *et al.*, 2005; Ruffner *et al.*, 2013) inoculated with 4×10^7 colony forming units per food pellet. For control treatments, pellets were treated with 10 µl sterile 0.9% NaCl solution. Instead of using petri dishes (Ruffner *et al.*, 2013), pellets were placed into Greiner six-well plates and presented to one larva per well. Five plates were prepared per bacterial strain (30 larvae per treatment). Larvae were incubated in the dark at room temperature and were fed with fresh, bacteria free diet when necessary. Survival rates were recorded daily. Larvae were considered to be dead when they did not react to repeated poking.

Bacterial colonization of *Spodoptera littoralis* larvae

Bacteria were extracted from surviving larvae at the end of the experiment. Larvae were surface-disinfested for 30 s in 70% ethanol, rinsed with sterile water and homogenized in 10 ml sterile 0.9% saline solution with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). Serial dilutions of the resulting homogenate were then plated onto King's B agar (King *et al.*, 1954) supplemented with ampicillin (40 µg ml^{-1}), chloramphenicol (13 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}) to select for the bacterial strains fed to the larvae. Plates for bacterial quantification were incubated for two days at 27°C before colony counting.

Identification of biocontrol activity

Biocontrol of *Pythium* damping-off of cucumber was assessed for each strain adapted after Sharifi-Tehrani *et al.* (1998). Aliquots of 200 µl of over-night cultures of bacteria grown in LB were plated on King's B agar (King *et al.*, 1954) and incubated for 24 h at 24°C. Bacteria were then scraped off the plate and washed in sterile distilled H_2O. Each bacterial strain was added to five pots filled with 120 g of TREF go PP7000 plant substrate (Gvz Rossat AG, Otelfingen, Switzerland) to a final concentration of 5×10^7 cfu per g soil. Each pot was inoculated with 0.3 g of *Pythium* inoculum grown on millet seeds and planted with three pre-germinated cucumber seeds. Pots were incubated at 70% humidity for 16 h with light (15 klux) at 22°C, followed by an 8-h dark period at 20°C. After 12 d, shoot weight per pot was recorded. Biocontrol activity was calculated after

Rezzonico *et al.* (2007) as: $(1 - ((W_C - W_I)/(W_C - W_P))) \times 100$ using shoot weight obtained in the control with neither bacterial nor pathogen inoculum (W_C), in the unprotected control with the pathogen alone (W_P) and in presence of the tested bacterial strain and the pathogen (W_I). A total of seven experiments was conducted and each strain was tested at least twice. The model strain *P. protegens* CHA0 was included as a reference in each experiment.

In vitro inhibition of plant pathogens

F. oxysporum Schlecht. f. sp. *radicis-lycopersici* strain Forl22 (Forl) and *P. ultimum* Trow strain 67-1 (Pu) were cultivated on malt agar as described by Sharifi-Tehrani *et al.* (1998). In vitro inhibition of Pu and Forl was assessed on malt agar (MA) and GCY (Tambong and Höfte, 2001) plates. Mycelial plugs were placed at the center of the plates either one day before (for Pu) or one day after (for Forl) adding the bacteria. Bacterial strains were grown overnight in LB. Cells were washed with sterile distilled H_2O and a suspension of an OD_{600} of 1.0 was prepared. The suspension was streaked out in a square around the mycelial plug using an inoculation loop. Plates were inoculated at 24°C. The mycelial diameter was measured after 2 d for Pu and after 8 d for Forl. Each strain was tested twice on each medium with four replicates.

Statistics

Data analysis was performed in R version 3.1.1. (http://www.r-project.org). Mortality rates of the insect toxicity tests with wild-type strains and data on in-vitro inhibition of plant pathogens were analysed by multiple comparisons using Kruskal-Wallis adjusted by Bonferroni-Holm. LT_{50} values were estimated based on the generalized linear model using the MASS package in R (Venables and Ripley, 2002). To test for significant differences in insect toxicity tests between *P. protegens* CHA0 and its mutant strains the Log-Rank test of the Survival package of R was used. To identify strains with significant biocontrol activity, a t-test was performed testing each strain against the respective unprotected control with pathogen alone.

Supplementary Results

Phylogeny of sequenced isolates

Many strains of the *Pseudomonas fluorescens* group were classified years ago, and their taxonomic status was not updated since then. We performed a comparative systematic study to correctly assign the isolates that were sequenced in this study to the phylogeny within the genus *Pseudomonas* (Mulet *et al.*, 2012). A recent study (Gomila *et al.*, 2015) has performed similar work with other published genomes, some of which were also included in our study.

The core genome tree (Figure 1), practically a core genome Multilocus Sequence Analysis (MLSA) (Blom *et al.*, 2009), confirmed the phylogenetic position of a range of isolates that we included in sequencing. For publicly available genomes, the position is corresponding to the study of Gomila *et al.* (2015), whereas we confirmed the position of some isolates that already had a unconfirmed status in phylogeny. The phylogenetic position of the isolates could now be confirmed using digital DNA-DNA hybridization (Supplementary Table S3)(Meier-Kolthoff *et al.*, 2014), average amino acid identities (Supplementary Table S3) of the core genome (Konstantinidis *et al.*, 2006) and the four-gene MLSA (Supplementary Figure S1) (Mulet *et al.*, 2012; Gomila *et al.*, 2015).

Five strains that were sequenced in this study are now included in *P. protegens* (Ramette *et al.*, 2011): strains CHA0^{T}, PGNR1, BRIP, K94.41 and PF, while two isolates (PCL1391 and CD), already included in *P. chlororaphis* (Chin-A-Woeng *et al.*, 1998; Ruffner *et al.*, 2015), can now be assigned to the respective subspecies as we also included three of the subspecies type strains in the genome analysis. Strain PCL1391 belongs to *P. chlororaphis* subsp. *piscium*, whereas strain CD is a *P. chlororaphis* subsp. *aureofaciens*. By searching the annotations for genes coding for the differential phenotypes as described in literature (Peix *et al.*, 2007; Burr *et al.*, 2010), we could confirm these designations.

Using the data generated in this study, strain TM1A3 is confirmed as member of the species *P. brassicacearum* by its relationship to type strain *P. brassicacearum* subsp. *brassicacearum* NFM421^{T}, but this strain cannot yet be assigned to a subspecies, as MLSA or genomic data for the other subspecies are missing. Based on AAI and DNA-DNA hybridization strains P12 and PITR2 are closely related to the type strains of *P. kilonensis and P. thivervalensis*, respectively, which were also sequenced in this study and can thus

be assigned to these species (Supplementary Table S3).

We cannot assign a species name to the other species. Three strains (Q12-87, P97.38 and Pf153) belong to the *P. corrugata* subgroup, while *Pseudomonas* sp. MIACH is included in the *P. fluorescens* subgroup (Supplementary Figure 1). This is in agreement with the core genome tree (Figure 1). *Pseudomonas* sp. P1.8 is, based on the MLSA, belonging to the *P. jessenii* subgroup, while *Pseudomonas* sp. P1.31 is a member of the *P. koreensis* subgroup. The closest related genome-sequenced strains included in Figure 1 were also assigned to the corresponding subgroups (Gomila *et al.*, 2015). However, none of these can be assigned to a known *Pseudomonas* species, indicating that these strains represent novel species within the genus.

Plant-beneficial effects and antifungal compounds

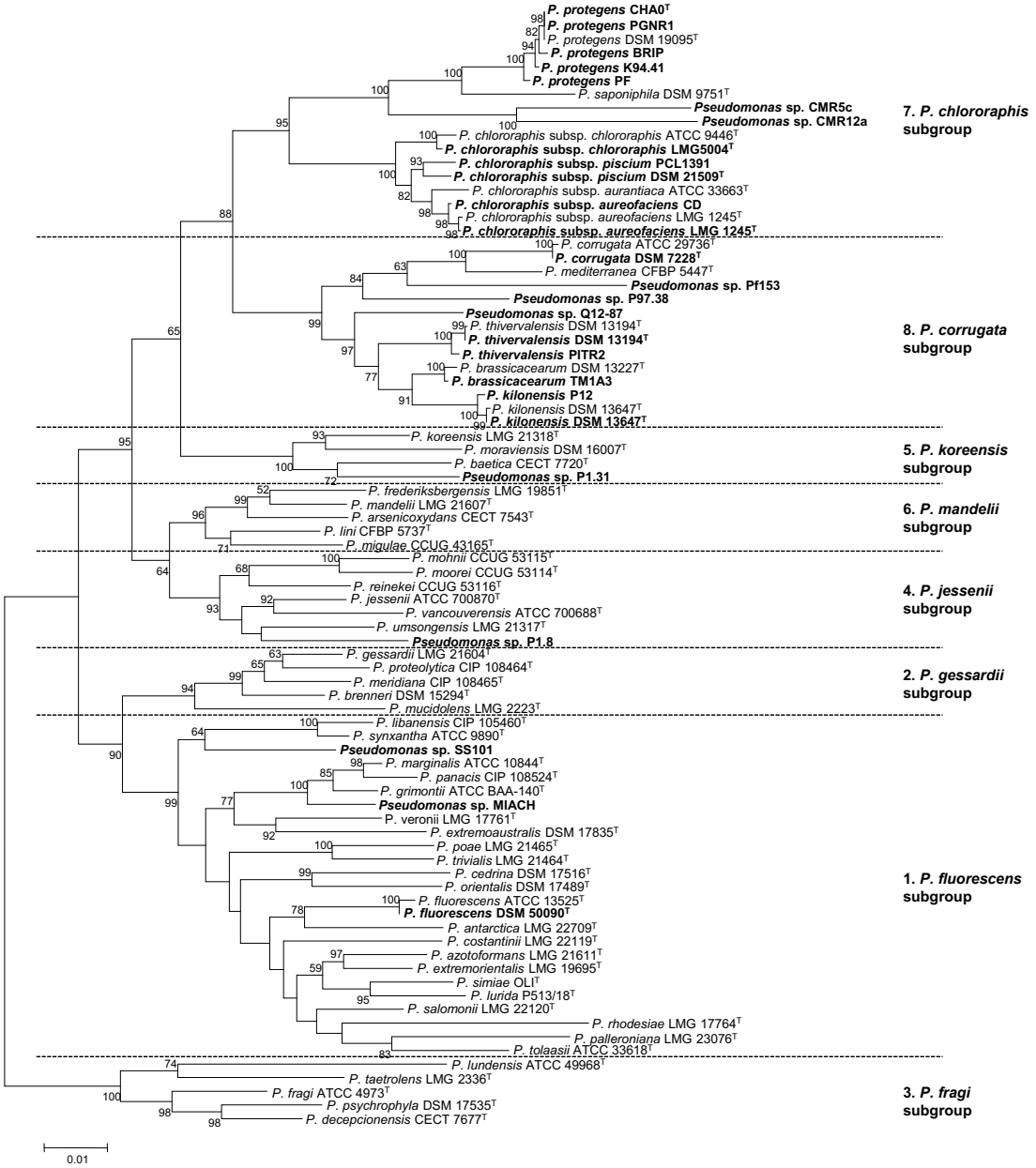
In a pot experiment all strains were tested for their biocontrol activity against the oomycete pathogen *Pythium ultimum* on cucumber roots. In all sub-clades, several strains were found to display effective plant protection whereas others had no significant biocontrol activity. Thus, biocontrol activity seems to be phylogenetically less predictable than insecticidal activity. The presence of biosynthetic genes for the two important antifungal metabolites 2,4-diacetylphloroglucinol (DAPG) (*phl*) and phenazine (Phz) (*phz*) was not necessarily linked to *P. ultimum* biocontrol since strains *P. chlororaphis* subsp. *aureofaciens* LMG 5004 (*phz*$^+$), *P. kilonensis* DSM 13647^T (*phl*$^+$), and *Pseudomonas* sp. CMR5c (*phz*$^+$, *phl*$^+$) did not provide any protection against the root pathogen (Figure 2). In contrast, all *P. protegens* strains displayed significant biocontrol ability in repeated experiments (Supplementary Table S5). The fact that the strains *Pseudomonas* sp. CMR5c and *P. kilonensis* P12 that were previously shown to have strong biocontrol activity (Keel *et al.*, 1996; Perneel *et al.*, 2007) did not protect cucumber plants against *P. ultimum* might be explained by the different experimental conditions used in this study, such as the plant as well as the pathogen species or the substrate.

Similar to the results on insecticidal activity, no connection between the original habitat and the degree of plant protection was observed. Thus, also the strains *P. chlororaphis* subsp. *piscium* DSM 21509^T or *P. protegens* BRIP recently isolated from fish and cyclops, respectively, provided significant biocontrol activity (Supplementary Table S5). In general, antifungal metabolite production appears to be less an adaptation to life on roots than a universal defence mechanism against microbial competitors. For instance

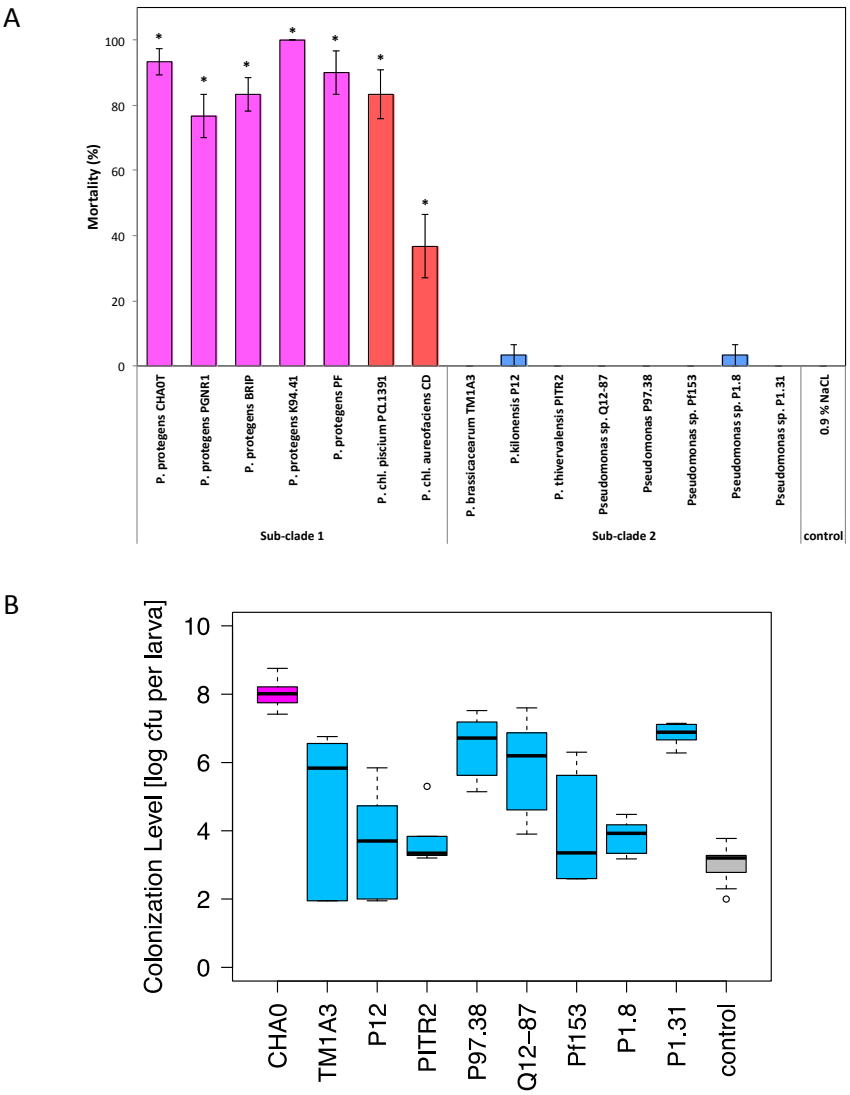
the fish isolate *P. chlororaphis* subsp. *piscium* DSM 21509^T was also found to have *in vitro* activity towards the oomycete fish pathogen *Saprolegnia parasitica,* which causes significant losses in fish hatcheries and breeding units (data not shown). Thus, an isolate from a certain habitat could also be used as biocontrol agent in a completely different ecological context.

A subset of 15 strains of sub-clade 1 and 2 was further tested for *in vitro* inhibition of mycelial growth of *P. ultimum* and a second plant pathogen, *Fusarium oxysporum*, on MA and GCY medium, favouring the production of DAPG or Phz, respectively (Figure 2, Supplementary Figure S3). Throughout both phylogenetic groups, all strains except P1.8 were found to exhibit *in vitro* pathogen inhibition with DAPG and Phz producing strains performing best on media conducive to metabolite biosynthesis.

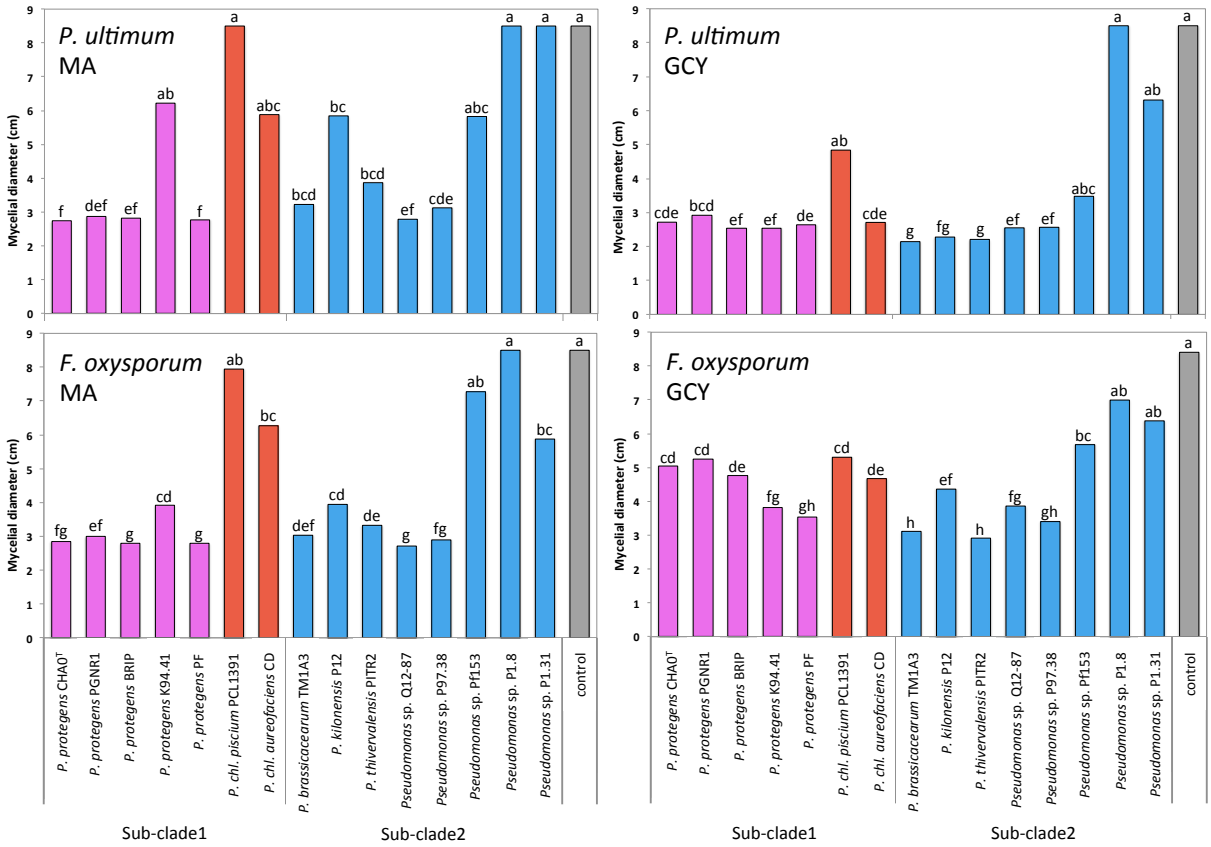
Supplementary Figures



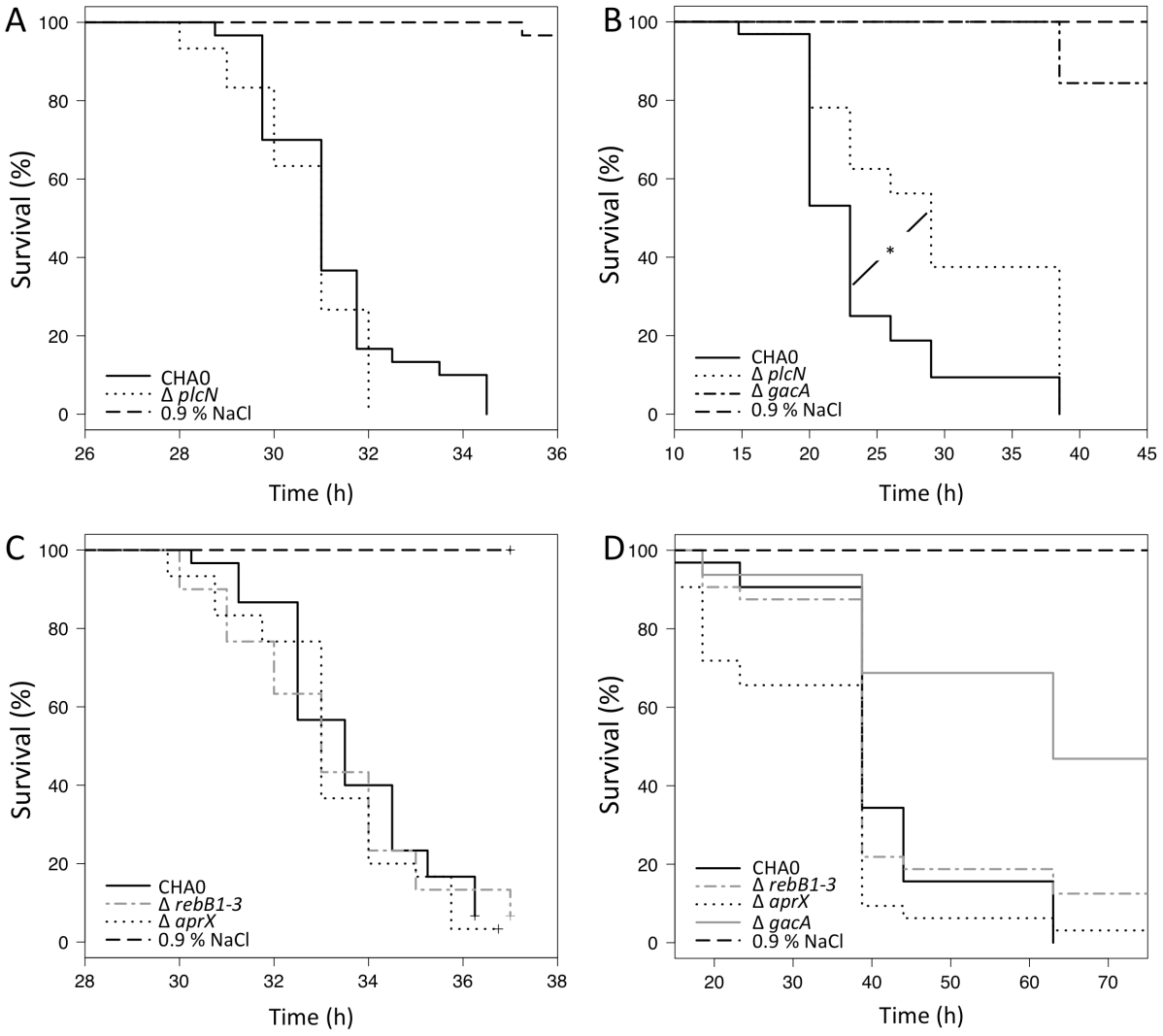
Supplementary Figure S1 Maximum Likelihood phylogenetic tree of strains belonging to the *P. fluorescens* group based on the four-gene (16S rRNA, *gyrB*, *rpoB* and *rpoD*) MLSA scheme of Mulet *et al.* (2012). Strains investigated in this study are indicated in bold. Bootstrap values over 50% are indicated in the tree.



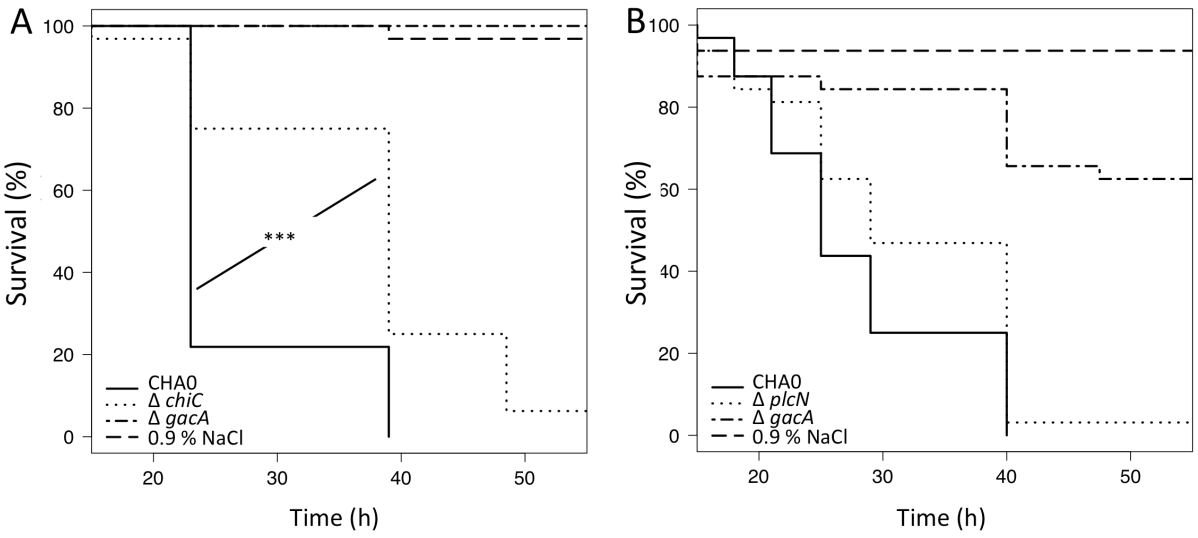
Supplementary Figure S2 Sub-clade 1 strains cause lethal oral infections in *Spodoptera littoralis* larvae while sub-clade 2 strains, although some of them are able to persist in the insect, do not kill the larvae. Survival **(A)** and colonization **(B)** rates of *S. littoralis* larvae upon feeding on artificial diet inoculated with either 4 x 10^7 cells of the indicated *Pseudomonas* strains or 0.9% NaCl (control). **(A)** Survival of larvae after 5 d. Bars show means (± se) of five replicates with six larvae each. Asterisks indicate bacterial treatments that were significantly different from the control based on multiple comparisons by Kruskal-Wallis adjusted by Bonferroni-Holm ($p \leq 0.05$). Each strain was tested in an independent second experiment with highly similar results. **(B)** Some strains of sub-clade 2 are able to persist in *S. littoralis* larvae whereas numbers of others strongly decline within a few days. To get an estimate of the capacity of inoculants to persist and multiply within *S. littoralis* larvae upon ingestion, six surviving larvae were extracted and colonization levels were assessed by plating serial dilutions on selective medium at the end of the experiment. Data derived from two independent experiments. Strains of sub-clade 1, here represented by *P. protegens* CHA0, generally multiply to levels of about 10^8 cfu/larva. Colonization levels in control larvae represent bacterial background levels on King's B agar (King *et al.*, 1954) supplemented with ampicillin (40 µg ml^{-1}), chloramphenicol (13 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}).



Supplementary Figure S3 Inhibition of mycelial growth of *Pythium ultimum* and *Fusarium oxysporum* on MA and GCY medium. Bacteria were streaked out in a square around a plug of oomycete/fungal mycelium and mycelial diameter was measured after 2 days for *P. ultimum* **(A)** and 8 days for *F. oxysporum* **(B)**. Strains with different letters were significantly different from each other based on multiple comparisons by Kruskal-Wallis adjusted by Bonferroni-Holm ($p \leq 0.05$). Each strain was tested in an independent second experiment with highly similar results.



Supplementary Figure S4 Deletion of *plcN* (encoding phospholipase C), but not of *aprX* or the *reb* cluster, reduces oral activity of *P. protegens* CHA0 against insect larvae. (A, C) Systemic activity against *Galleria mellonella*. 30 larvae per treatment were injected with 2 x 10^3 bacterial cells and survival was recorded every hour. **(B, D)** Oral activity against *Plutella xylostella*. Larvae were fed on artificial diet inoculated with 4 x 10^6 bacterial cells. **(B)** The virulence of the phospholipase C-negative (Δ*plcN*) mutant was slightly reduced compared to the wild type strain (p-value<0.01, Log-Rank test, Survival Package in R). Although the effect was not significant in all experiments, the tendency of slower killing was always observed. Each mutant was tested at least three times with similar results. One repetition is depicted in Supplementary Figure S5. CHA0, wild type; Δ*plcN*, phospholipase *C*-negative mutant; Δ*gacA*, GacA-negative mutant; Δ*rebB1-3*, mutant for the *rebB*-cluster; Δ*aprX*, metallopeptidase AprX-negative mutant; 0.9% NaCl served as negative control.



Supplementary Figure S5 Repetition of experiments depicted in Figure 4 and Supplementary Figure S4. Oral activity against *Plutella xylostella*. Larvae were exposed to artificial diet inoculated with 4 x 10^6 bacterial cells. **(A)** Significant differences according to a Log-Rank test (Survival Package in R) between treatments with the wild type CHA0 and the chitinase C-negative mutant are indicated with ***(p-value <0.0001). CHA0, wild type; Δ*chiC*, chitinase C-negative mutant; Δ*gacA*, GacA-negative mutant; Δ*plcN*, phospholipase C-negative mutant; 0.9% NaCl served as negative control.

Supplementary Tables

Supplementary Table S1 Full list of gene clusters associated with biocontrol or insecticidal activity for all strains shown in Figure 1. Supplementary to overview Figure 2.

		Gene or metabolite name (Locus tag)																					
Accession number	Strain	*fit* PFL_2980-PFL_2987	*chiC* PCL1391_1854-PCL1391_1855	chitinase PCL1391_3057-PCL1391_3058	*plcN* PCL1391_2966	*aprX* PCL1391_2141	*aprA* PCL1391_3021	*psl* PCL1391_4983,4985-4994	*rebB* PCL1391_0072, 0073_0075_0076		DAPG PFL_5951-PFL_5958	PCA PCL1391_4880-PCL1391_4888	PCN PCL1391_4889	2-OH-PCA PchlO6_5227	HCN PFL_2577-PFL_2579	PRN PFL_3604-PFL_3607	PLT PFL_2784-PFL_2800	HPR PchlO6_4242-PchlO6_4244	rhizoxin PFL_2989-PFL_2997	CLP PFL_2145-PFL_2147
CP003190.1	*P. protegens* CHA0T	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	-	+
LHUV00000000	*P. protegens* PGNR1	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	-	+
LHUW00000000	*P. protegens* BRIP	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	-	+
LHUU00000000	*P. protegens* K94.41	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	-	+
CP000076.1	*P. protegens* Pf-5	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	+	+
LHUX00000000	*P. protegens* PF	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	+	+
AP014522.1	*P. protegens* Cab57	+	+	-	+	+	+	+	+		+	-	-	-	+	+	+	-	-	+
LHUY00000000	*Pseudomonas* sp. CMR5c	+	+	-	+	+	+	+	+		+	+	+	-	+	+	+	-	-	+
LHUZ00000000	*P. chlororaphis* subsp. *piscium* JF3835T	+	+	+	+	+	+	+	+		-	+	+	-	+	-	-	-	-	+
ATBG00000000	*P. chlororaphis* HT66	+	+	+	+	+	+	+	+		-	+	+	-	+	-	-	-	-	+
LFUT00000000	*P. chlororaphis* subsp. *piscium* PCL1391	+	+	+	+	+	+	+	+		-	+	+	-	+	-	-	-	-	+
AYUD00000000.1	*P. chlororaphis* subsp. *aurantiaca* PB-St2	±	+	+	+	-	+	+	±		-	+	-	+	+	+	-	+	-	-
CP009290.1	*P. chlororaphis* subsp. *aurantiaca* JD37	±	+	+	+	+	+	+	+		-	+	-	+	+	+	-	+	-	-
CP008696.1	*P. chlororaphis* PA23	+	+	+	+	+	+	+	+		-	+	-	+	+	+	-	+	-	-
LHVB00000000	*P. chlororaphis* CD	+	+	+	+	+	+	+	+		-	+	-	+	+	+	-	+	-	-
LHVA00000000	*P. chlororaphis* subsp. *aureofaciens* LMG 1245T	+	+	±	+	+	+	+	+		-	+	-	+	+	+	-	+	-	-
AWWJ00000000	*P. chlororaphis* YL-1	+	+	+	+	+	+	+	±		-	+	-	+	+	+	-	+	-	-
CM001490.1	*P. chlororaphis* O6	+	+	+	+	+	+	+	+		-	+	-	+	+	+	-	+	-	-
CM001559.1	*P. chlororaphis* subsp. a*ureofaciens* 30-84	+	+	+	+	+	+	+	+		-	+	-	+	+	+	-	+	-	-
LHVC00000000	*P. chlororaphis* subsp. *chlororaphis* LMG 5004T	+	+	+	+	+	+	+	+		-	+	+	-	+	+	-	+	-	-
AFOY00000000	*Pseudomonas* sp. HK44	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
LHVD00000000	*P. brassicacearum* TM1A3	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
AZOC00000000.1	*P. brassicacearum* 51MFCVI2.1	-	-	-	-	-	-	-	-		-	-	-	-	+	-	-	-	-	-
CP002585.1	*P. brassicacearum* subsp. *brassicacearum* NFM421	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
AHPO00000000	*P. brassicacearum* Q8r1-96	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
LHVH00000000	*P. kilonensis* DSM 13647T	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
LHVG00000000	*P. kilonensis* P12	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
CP003150.1	*Pseudomonas* sp. F113	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
LHVE00000000	*P. thivervalensis* DSM13194T	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
LHVF00000000	*P. thivervalensis* PITR2	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
LHVI00000000	*Pseudomonas* sp. Q12-87	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
AGBM00000000	*Pseudomonas* sp. Q2-87	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
CP007410.1	*Pseudomonas* sp. DF41	-	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
LHVJ00000000	*Pseudomonas* sp. P97.38	-	-	-	-	-	+	-	-		+	-	-	-	+	-	-	-	-	-
ATKI00000000	*P. corrugata* CFBP5454	-	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
LHVK00000000	*P corrugata* DSM7228 T	-	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
AUPB00000000.1	*P. mediterranea* CFBP 5447	-	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
LHVL00000000	*Pseudomonas* sp. Pf153	-	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
CP003880.1	*Pseudomonas* sp. UW4	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
LHVM00000000	*Pseudomonas* sp. P1.8	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
ARLP00000000	*P. mandelii* 36MFCvi1.1	±	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
AZQQ00000000	*P. mandelii* PD30	-	-	-	-	-	+	-	-		-	-	-	-	-	-	-	-	-	-
CP005960.1, CP005961.1	*P. mandelii* JR-1	-	-	+	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
CP000094.2	*Pseudomonas* sp. Pf0-1	-	-	+	-	+	+	-	-		-	-	-	-	+	-	-	-	-	+
LHVN00000000	*Pseudomonas* sp. P1.31	-	-	-	-	-	+	-	-		-	-	-	-	+	-	-	-	-	-
ALYL00000000	*Pseudomonas* sp. R124	-	-	+	-	+	+	-	-		-	-	-	-	+	-	-	-	-	+
CP008896.1	*Pseudomonas* sp. UK4	-	-	-	-	-	+	+	-		-	-	-	-	-	-	-	-	-	-
JENC00000000.1	*Pseudomonas* sp. ATCC 17400	-	-	-	-	-	+	+	-		-	-	-	-	-	-	-	-	-	-
AKXH00000000	*Pseudomonas* sp. BBc6R8	-	-	-	+	-	+	±	-		-	-	-	-	-	-	-	-	-	-
CP004045.1	*P. poae* RE*1-1-14	-	-	-	-	-	+	±	-		-	-	-	-	-	-	-	-	-	+
AMZW01000000	*P. poae* BRIP34879	-	-	-	-	-	+	±	-		-	-	-	-	-	-	-	-	-	+
AOUH00000000	*P. veronii* 1YdBTEX2	±	-	-	-	-	+	-	-		-	-	-	-	-	-	-	-	-	-
CP006852.1	*Pseudomonas* sp. TKP	-	-	-	-	-	+	±	-		-	-	-	-	-	-	-	-	-	-
LHVP00000000	*P. fluorescens* DSM50090T	-	-	+	-	-	+	±	-		-	-	-	-	-	-	-	-	-	+
LHVO00000000	*Pseudomonas* sp. MIACH	-	-	-	-	-	+	±	-		-	-	-	-	-	-	-	-	-	+
NC_012660.1	*Pseudomonas* sp. SBW25	-	-	-	-	-	+	±	-		-	-	-	-	-	-	-	-	-	+
AVQG00000000	*Pseudomonas* sp. EGD-AQ6	-	-	-	-	-	+	±	-		-	-	-	-	-	-	-	-	-	-
CM001025.1	*Pseudomonas* sp. WH6	-	-	-	-	-	+	-	-		-	-	-	-	-	-	-	-	-	-
AMZG00000000.1	*Pseudomonas* sp. BS2	-	-	-	-	-	+	-	-		-	-	+	-	-	-	-	-	-	-
CM001514.1	*P. synxantha* BG33R	-	-	+	-	-	+	±	±		-	-	-	-	-	-	-	-	-	+
CM001513.1	*Pseudomonas* sp. SS101	-	-	+	-	-	+	±	±		-	-	-	-	-	-	-	-	-	+
AOJA00000000	*Pseudomonas* sp. FH5	-	-	+	-	-	+	±	±		-	-	-	-	-	-	-	-	-	-
NC_017911.1; NC_021361.1	*Pseudomonas* sp. A506	-	-	+	-	-	+	±	±		-	-	-	-	-	-	-	-	-	-
AHZX00000000.1	*P. fragi* B25	-	-	-	-	+	-	-	-		-	-	-	-	±	-	-	-	-	-

Loci were defined as being present when showing 70% similarity over 70% of gene length to the loci indicated in the table. As none of the strains harbors all loci, three different reference strains (PCL1391, Pf-5, O6) were used. +, gene/s present; -, gene/s absent; ±, gene cluster partially present. As most genomes consist of several contigs, genes might be found to be absent in a certain strain although they are in fact present, but are located at the border of contigs. Abbreviations: *P. fluorescens* insecticidal toxin-cluster (*fit*); chitinase C (*chiC*); phospholipase C (*plcN*); metallopeptidase *aprX* (*aprX*); alkaline metalloprotease *aprA* (*aprA*); *rebB*-cluster (*rebB*); *psl*-cluster (*psl*); 2,4-diacetylphloroglucinol (DAPG); phenazines: phenazine-1-carboxamide (PCN), phenazine-1-carboxylic acid (PCA), 2-hydroxy-PCA (2-OH-PCA); hydrogen cyanide (HCN); pyrrolnitrin (Prn), pyoluteorin (Plt); 2-hexyl-5-propyl-alkylresorcinol (HPR); cyclic lipopeptide (CLP).

Supplementary Table S2 Strains, plasmids and primers used in this study

Name	Relevant characteristics [1] or sequence (5′ → 3′) [2]	Reference or comment
Pseudomonas protegens		
CHA0	Wild type	(Stutz *et al.*, 1986; Jousset *et al.*, 2014)
CHA5099	Δ*chiC* (deletion of PFLCHA0_c21380)	This study
CHA5221	Δ*rebB1-3* (deletion of PFLCHA0_c01820 through PFLCHA0_c01860)	This study
CHA5222	Δ*aprX* (deletion of PFLCHA0_c25470)	This study
CHA5223	Δ*plcN* (deletion of PFLCHA0_c31570)	This study
Escherichia coli		
DH5α, DH5α λpir	Laboratory strains	(Sambrook and Russel, 2001)
Plasmids		
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r, Ap^r	(Martinez-Garcia and de Lorenzo, 2011)
pME8327	pEMG-Δ*chiC*; suicide plasmid for the in-frame deletion of PFLCHA0_c21380 (*chiC*) in CHA0; Km^r	This study
pME11026	pEMG-Δ*rebB1-3*; suicide plasmid for the deletion of the PFLCHA0_c01820 to PFLCHA0_c01860 region (*rebB1-3* cluster) in CHA0; Km^r	This study
pME11027	pEMG-Δ*aprX*; suicide plasmid for the in-frame deletion of PFLCHA0_c25470 (*aprX*) in CHA0; Km^r	This study
pME11028	pEMG-Δ*plcN*; suicide plasmid for the in-frame deletion of PFLCHA0_c31570 (*plcN*) in CHA0; Km^r	This study
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	(Martinez-Garcia and de Lorenzo, 2011)
Primers		
aprX-del-1	GGAATTCGATGGGCCTGTTCTGAGAGG, EcoRI	Deletion of CHA0 *aprX*
aprX-del-2	CCCAAGCTTTGCTTCCGAGAGTGCTTTTGAC, HindIII	Deletion of CHA0 *aprX*
aprX-del-3	CCCAAGCTTAGCCTGATGATCGACCTGAC, HindIII	Deletion of CHA0 *aprX*
aprX-del-4	CGGGATCCTACCAGCAGTTCTGCAACCAG, BamHI	Deletion of CHA0 *aprX*
chiD-1	CGGAATTCGCCACAGGCTCAACTAAAACAT, EcoRI	Deletion of CHA0 *chiC*
chiD-2	GGGGTACCAATGCTCGGCATCAGGGAAGCA, KpnI	Deletion of CHA0 *chiC*
chiD-3	GGGGTACCCATGGCTGAGTTGTGACGGCCA, KpnI	Deletion of CHA0 *chiC*
chiD-4	CGGGATCCCGCTTACCAATGATTACAACTG, BamHI	Deletion of CHA0 *chiC*
plcC-del-1	GGAATTCATAACGCCACCCATTTCAGC, EcoRI	Deletion of CHA0 *plcN*
plcC-del-2	CCCAAGCTTACTGGGCATGGGTTATTGAGTC, HindIII	Deletion of CHA0 *plcN*
plcC-del-3	CCCAAGCTTGCATGAAGACCTTGGCAAAAATG, HindIII	Deletion of CHA0 *plcN*
plcC-del-4	CGGGATCCGGCCTATGCACGAAAGTTGT, BamHI	Deletion of CHA0 *plcN*
reb-del-1	GGAATTCGTATTGCCCGGTTTGCAGC, EcoRI	Deletion of CHA0 *reb* cluster
reb-del-2	CCCAAGCTTACTGGGCATGGGTTATTGAGTC, HindIII	Deletion of CHA0 *reb* cluster
reb-del-3	CCCAAGCTTGCATGAAGACCTTGGCAAAAATG, HindIII	Deletion of CHA0 *reb* cluster
reb-del-4	CGGGATCCCGCTTACCAATGATTACAACTG, BamHI	Deletion of CHA0 *reb* cluster

[1] Ap^r, ampicillin; Gm^r, gentamicin; and Km^r, kanamycin resistance, respectively. [2] Specified restriction sites are underlined.

Supplementary Table S3 Mean amino acid identities (AAI) and Genome-to-Genome Distance Calculator (GGDC) values for all genomes related to *P. brassicacearum*, *P. kilonensis* and *P. thivervalensis*.

A AAI values

		1	2	3	4	5	6	7	8	9	10	11	12
1	*P. brassicacearum* subsp. *brassicacearum* NFM 421^{T}		99.82	99.81	99.85	97.74	97.74	97.82	96.44	96.39	94.67	94.67	94.87
2	*P. brassicacearum* TM1A3	99.82		99.79	99.84	97.73	97.73	97.82	96.44	96.38	94.66	94.67	94.85
3	*P. brassicacearum* 51MCFVI21	99.81	99.79		99.81	97.72	97.72	97.78	96.42	96.37	94.66	94.65	94.84
4	*P. fluorescens* Q8r1-96	99.85	99.84	99.81		97.73	97.73	97.82	96.43	96.38	94.66	94.65	94.86
5	*P. kilonensis* DSM 13647^{T}	97.74	97.73	97.72	97.73		99.49	98.33	96.61	96.59	94.54	94.54	94.73
6	*P. kilonensis* P12	97.74	97.73	97.72	97.73	99.49		98.33	96.61	96.57	94.50	94.50	94.72
7	*P. fluorescens* F113	97.82	97.82	97.78	97.82	98.33	98.33		96.62	96.58	94.58	94.58	94.71
8	*P. thivervalensis* DSM 13194^{T}	96.44	96.44	96.42	96.43	96.61	96.61	96.62		99.48	94.43	94.43	94.41
9	*P. thivervalensis* PITR2	96.39	96.38	96.37	96.38	96.59	96.57	96.58	99.48		94.40	94.40	94.37
10	*Pseudomonas* sp. Q12-87	94.67	94.66	94.66	94.66	94.54	94.50	94.58	94.43	94.40		99.79	94.48
11	*P. fluorescens* Q2-87	94.67	94.67	94.65	94.65	94.54	94.50	94.58	94.44	94.40	99.79		94.47
12	„*P. brassicacearum*“ DF41	94.87	94.85	94.84	94.86	94.73	94.72	94.71	94.41	94.37	94.48	94.47	

B GGDC values

		1	2	3	4	5	6	7	8	9	10	11	12
1	*P. brassicacearum* subsp. *brassicacearum* NFM 421^{T}		96.0	96.9	96.6	59.1	59.1	61.0	46.2	45.9	36.5	36.7	38.6
2	*P. brassicacearum* TM1A3	96.0		96.3	96.1	59.2	59.1	60.9	46.1	46.0	36.3	36.4	38.4
3	*P. brassicacearum* 51MCFVI21	96.8	96.3		97.0	59.1	59.2	61.1	46.2	46.0	36.5	36.5	38.4
4	*P. fluorescens* Q8r1-96	96.6	96.1	97.0		59.0	58.9	60.9	46.1	46.0	36.6	36.7	38.5
5	*P. kilonensis* DSM 13647^{T}	59.1	59.2	59.1	59.0		88.5	64.5	47.4	47.0	36.4	36.5	38.8
6	*P. kilonensis* P12	59.1	59.1	59.2	58.9	88.5		64.5	47.2	46.9	36.5	36.5	38.3
7	*P. fluorescens* F113	61.0	60.9	61.1	60.9	64.5	64.5		46.7	46.3	36.2	36.3	38.4
8	*P. thivervalensis* DSM 13194^{T}	46.2	46.1	46.2	46.1	47.4	47.2	46.7		88.0	35.4	35.5	37.2
9	*P. thivervalensis* PITR2	45.9	46.0	46.0	46.0	47.0	46.9	46.3	88.0		35.3	35.3	37.0
10	*Pseudomonas* sp. Q12-87	36.5	36.3	36.5	36.6	36.4	36.5	36.2	35.4	35.3		96.0	37.1
11	*P. fluorescens* Q2-87	36.7	36.4	36.5	36.7	36.5	36.5	36.3	35.5	35.3	96.0		37.2
12	„*P. brassicacearum*“ DF41	38.6	38.4	38.4	38.5	38.8	38.3	38.4	37.2	37.0	37.1	37.2	

Supplementary Table S4 Lethal time (LT_{50}) and survival of *Plutella xylostella* larvae upon oral uptake of *Pseudomonas* strains.

Sub-clade	Strain	LT50 (d)		survival (%) at 3 dpi ±sdev			
Sub-clade 1	*P. protegens* CHA0^T	1.6	(1.5; 1.8) ^{abc}	3.1	±	6.3	*
	P. protegens PGNR1	1.6	(1.4; 1.8) ^{abc}	0.0	±	0.0	*
	P. protegens BRIP	1.3	(1.1; 1.5) ^{a}	0.0	±	0.0	*
	P. protegens K94.41	1.0	(-19.1; 21.2) ^{abcdef}	0.0	±	0.0	*
	P. protegens PF	1.4	(1.2; 1.6) ^{ab}	0.0	±	0.0	*
	Pseudomonas sp. CMR12a	1.9	(1.7; 2.2) ^{cde}	6.3	±	7.2	*
	P. chl. piscium DSM 21509^T	1.6	(1.4; 1.8) ^{abc}	3.1	±	6.3	*
	P. chl. piscium PCL1391	1.7	(1.5; 1.9) ^{bcd}	0.0	±	0.0	*
	P. chl. aureofaciens CD	1.5	(1.3; 1.7) ^{ab}	3.1	±	6.3	*
	P. chl. aureofaciens LMG 1245^T	2.1	(1.9; 2.4) ^{def}	21.9	±	12.0	*
	P. chl. chlororaphis LMG 5004^T	2.4	(2.1; 2.8) ^{ef}	31.3	±	12.5	*
Sub-clade 2	*P. brassicacearum* TM1A3	NA		81.3	±	21.7	
	P. kilonensis DSM 13647^T	NA		93.8	±	7.2	
	P. kilonensis P12	NA		87.5	±	10.2	
	P. thivervalensis DSM 13194^T	NA		84.4	±	12.0	
	P. thivervalensis PITR2	NA		90.6	±	12.0	
	Pseudomonas sp. Q12-87	NA		81.3	±	7.2	
	Pseudomonas sp. P97.38	NA		87.5	±	17.7	
	P. corrugata DSM 7228^T	NA		75.0	±	21.7	
	Pseudomonas sp. Pf153	NA		93.8	±	7.2	
	Pseudomonas sp. P1.8	NA		87.5	±	10.2	
	Pseudomonas sp. P1.31	NA		84.4	±	12.0	
Sub-clade 3	*P. fluorescens* DSM 50090^T	NA		65.6	±	12.0	*
	Pseudomonas sp. MIACH	2.7	(1.8; 3.5) ^{def}	45.8	±	26.0	*
	Pseudomonas sp. SS101	2.8	(2.3; 3.3) ^{f}	46.9	±	27.7	*
control	0.9% NaCL	NA		96.9	±	6.3	

Repetition of the experiment depicted in Figure 3 and Table 2. *Plutella xylostella* larvae were exposed to food pellets inoculated with 8 x 10^7 bacterial cells. LT_{50} values are estimates based on the generalized linear model using the MASS package in R (Venables and Ripley, 2002). Numbers in brackets depict 95% confidence intervals for LT_{50} and significantly different values within the same column are followed by different letters. NA = no LT_{50} value was calculated, because end mortality was less than 50%. Asterisks indicate significant differences compared to control larvae treated with 0.9% NaCl based on multiple comparisons by Kruskal-Wallis adjusted by Bonferroni-Holm ($p \leq 0.05$).

Supplementary Table S5 Biocontrol activity against *Pythium ultimum* on cucumber plants

Sub-clade	Strain	Biocontrol Activity relative to *P. protegens* CHA0					
		repetition 1			repetition 2		
Sub-clade 1	*P. protegens* CHA0^T	1.00		*	1.00		*
	P. protegens PGNR1	1.02	± 0.14	*	0.76	± 0.05	*
	P. protegens BRIP	0.94	± 0.24	*	1.07	± 0.09	*
	P. protegens K94.41	0.43	± 0.21	*	0.50	± 0.23	*
	P. protegens PF	0.35	± 0.16	*	0.49	± 0.33	*
	Pseudomonas sp. CMR5c	0.02	± 0.04		0.00	± 0.00	
	Pseudomonas sp. CMR12a	0.51	± 0.25	*	0.00	± 0.00	
	P. chl. piscium DSM 21509^T	0.54	± 0.28	*	0.21	± 0.30	
	P. chl. piscium PCL1391	0.72	± 0.15	*	0.55	± 0.30	*
	P. chl. aureofaciens LMG 1245^T	0.15	± 0.23		0.39	± 0.56	
	P. chl. aureofaciens CD	0.89	± 0.11	*	0.51	± 0.11	*
	P. chl. chlororaphis LMG 5004^T	0.00	± 0.00		0.00	± 0.00	
Sub-clade 2	*P. brassicacearum* TM1A3	0.72	± 0.22	*	0.31	± 0.31	
	P. kilonensis DSM 13647^T	0.00	± 0.00		0.00	± 0.00	
	P. kilonensis P12	0.23	± 0.29		0.18	± 0.20	
	P. thivervalensis DSM 13194^T	0.31	± 0.30		0.28	± 0.28	
	P. thivervalensis PITR2	0.70	± 0.15	*	0.74	± 0.07	*
	Pseudomonas sp. Q12-87	0.67	± 0.21	*	0.58	± 0.32	*
	Pseudomonas sp. P97.38	0.61	± 0.12	*	0.51	± 0.17	*
	P. corrugata DSM 7228^T	0.00	± 0.00		0.06	± 0.14	
	Pseudomonas sp. Pf153	0.52	± 0.12	*	0.25	± 0.38	
	Pseudomonas sp. P1.8	0.05	± 0.12		0.33	± 0.34	
	Pseudomonas sp. P1.31	0.61	± 0.11	*	0.54	± 0.21	*
Sub-clade 3	*P. fluorescens* DSM 50090^T	0.00	± 0.00		0.00	± 0.00	
	Pseudomonas sp. MIACH	0.39	± 0.47		1.02	± 0.47	*
	Pseudomonas sp. SS101	0.82	± 0.25	*	0.00	± 0.00	

Biocontrol activity was calculated after Rezzonico *et al.* (2007) as: $(1 - ((W_C - W_I)/(W_C - W_P))) \times 100$ using shoot weight obtained in the control with neither bacterial nor pathogen inoculum (W_C), in the unprotected control with the pathogen alone (W_P) and in presence of the tested bacterial strain and the pathogen (W_I). Due to the large number of strains, not all strains could be tested in the same experiment. Therefore, biocontrol activity is shown relative to the biocontrol activity of our model strain *P. protegens* CHA0, which was included as a reference in all experiments. A total of seven experiments was performed and each strain was tested at least twice (repetition 1 and repetition 2). Biocontrol activity for *P. protegens* CHA0 ranged between 63% and 100%. Means of five replicates ± sdev are shown. Statistics was performed for each experiment separately on absolute biocontrol activity values. Asterisks indicate that strains displayed significant biocontrol activity based on a t-test ($p = 0.05$) against the unprotected control with pathogen alone (W_P).

Supplementary Table S6 Genomic features

Sub-clade	Strain	# Reads * 10^6	# Contigs	Genome size (Mbp)	N50 (kb)	Coverage
Sub-clade 1	*P. protegens* PGNR1	1.96	15	6.86	871	75
	P. protegens BRIP	1.12	19	6.89	701	44
	P. protegens K94.41	1.28	17	6.99	582	50
	P. protegens PF	1.41	14	7.07	1051	52
	Pseudomonas sp. CMR5c	22.35	44	6.76	502	37
	P. chlororaphis subsp. *piscium* DSM21509^T	1.35	36	7.04	414	51
	P. chlororaphis subsp. *piscium* PCL1391	1.29	17	6.86	820	51
	P. chlororaphis subsp. *aureofaciens* LMG 1245^T	1.43	45	7.02	311	54
	P. chlororaphis subsp. *aureofaciens* CD	1.92	32	6.8	388	75
	P. chlororaphis subsp. *chlororaphis* LMG 5004^T	1.13	15	6.79	875	44
Sub-clade 2	*P. brassicacearum* TM1A3	2.16	29	6.69	552	86
	P. kilonensis DSM 13647^T	1.60	44	6.39	281	66
	P. kilonensis P12	1.98	44	6.39	277	81
	P. thivervalensis DSM 13194^T	1.65	25	6.58	445	67
	P. thivervalensis PITR2	1.75	26	6.77	661	68
	Pseudomonas sp. Q12-87	1.33	45	6.30	261	56
	Pseudomonas sp. P97.38	2.12	36	6.06	278	92
	P. corrugata DSM 7228^T	1.63	31	6.13	374	71
	Pseudomonas sp. Pf153	1.70	30	5.98	577	75
	Pseudomonas sp. P1.8	1.87	43	6.36	325	79
	Pseudomonas sp. P1.31	2.18	48	6.27	262	92
Sub-clade 3	*P. fluorescens* DSM 50090^T	1.43	17	6.39	973	59
	Pseudomonas sp. MIACH	1.37	73	6.82	236	54

Supplementary Table S7. Genes specific to insecticidal strains. Locus tags (prefix PCL1391_) and gene names are indicated for *Pseudomonas chlororaphis* subsp. *piscium* PCL1391.

Locus Tags	Gene	*P. protegens*	*Pseudomonas* sp. CMR	*P. chlororaphis*	*P. fluorescens* SS101	*P. fluorescens* DSM50090^T	*Pseudomnas* sp. MIACH	sub-clade 2
PCL1391_0010	Thioesterase	+	+	+	-	-	-	-
PCL1391_0029	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_0030	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_0072	RebB like protein	+	+	+	+	-	-	-
PCL1391_0073	Hypothetical protein	+	+	+	+	-	-	-
PCL1391_0075	RebB protein	+	+	+	+	-	-	-
PCL1391_0076	RebB protein	+	+	+	+	-	-	-
PCL1391_0101	Hypothetical protein	+	+	+	+	+	-	-
PCL1391_0108	Cyanate transport protein CynX	+	+	+	+	+	+	-
PCL1391_0109	CMP deaminase	+	+	+	+	+	-	-
PCL1391_0110	Putative ankyrin-containing lipoprotein	+	+	+	-	-	-	-
PCL1391_0111	LysR family transcriptional regulator	+	+	+	+	+	+	-
PCL1391_0170	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_0171	Polysaccharide deacetylase	+	+	+	-	-	-	-
PCL1391_0195	Ketosteroid isomerase	+	+	+	-	-	-	-
PCL1391_0279	Histidine-specific permease	+	+	+	+	+	+	-
PCL1391_0332	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_0603	Kynurenine formamidase	+	+	+	+	+	+	-
PCL1391_0604	Tryptophan 2,3-dioxygenase	+	+	+	+	+	+	-
PCL1391_0605	Aromatic amino acid transport protein AroP	+	+	+	+	+	+	-
PCL1391_0610	AsnC family transcriptional regulator	+	+	+	+	+	+	-
PCL1391_0611	Kynureninase	+	+	+	+	+	+	-
PCL1391_0612	Amino acid permease	+	+	+	+	+	+	-
PCL1391_0639	Alpha/beta hydrolase	+	+	+	-	-	-	-
PCL1391_0640	membrane protein	+	+	+	+	+	+	-
PCL1391_0733	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_0734	Phosphatidylcholine hydrolyzing phospholipase	+	+	+	+	+	+	-
PCL1391_0828	Serine transporter	+	+	+	+	+	-	-
PCL1391_0938	Signal transduction histidine kinase	+	+	+	+	-	-	-
PCL1391_0939	Hisitdine kinase	+	+	+	+	-	-	-
PCL1391_0940	LuxR family transcriptional regulator	+	+	+	+	+	+	-
PCL1391_0949	GNAT family acetyltransferase	+	+	+	-	-	-	-
PCL1391_1183	Membrane protein	+	+	+	-	-	-	-
PCL1391_1217	TonB-denpendent receptor	+	+	+	+	+	+	-
PCL1391_1218	Iron dicitrate transporter FecR	+	+	+	+	+	+	-
PCL1391_1219	RNA polymerase sigma factor	+	+	+	+	+	+	-
PCL1391_1245	Oxidoreductase	+	+	+	-	-	-	-
PCL1391_1247	GNAT family acetyltransferase	+	+	+	-	-	-	-
PCL1391_1251	Cyclic diguanylate phosphodiesterase	+	+	+	-	-	-	-
PCL1391_1352	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_1354	LysR family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_1370	AraC family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_1510	Endoribonuclease L-PSP	+	+	+	+	-	-	-
PCL1391_1588	Probable sugar efflux transporter	+	+	+	+	+	+	-
PCL1391_1733	Extradiol dioxygenase	+	+	+	-	+	+	-
PCL1391_1817	MFS transporter	+	+	+	-	-	-	-

PCL1391_1854	Chitin-binding protein	+	+	+	+	+	-	-
PCL1391_1855	Chitinase	+	+	+	+	+	-	-
PCL1391_1901	HxlR family transcriptional regulator	+	+	+	+	-	+	-
PCL1391_1903	Heme transporter CcmD	+	+	+	-	-	-	-
PCL1391_1904	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_1905	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_1906	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_1910	Haloacid dehalogenase	+	+	+	+	+	+	-
PCL1391_1978	IclR family transcriptional regulator	+	+	+	+	-	-	-
PCL1391_1979	ABC transporter substrate-binding protein	+	+	+	+	-	-	-
PCL1391_1980	Amino acid ABC transporter permease	+	+	+	+	-	-	-
PCL1391_1982	FAD-dependent oxidoreductase	+	+	+	+	-	-	-
PCL1391_2008	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_2011	RNA-binding protein	+	+	+	-	-	-	-
PCL1391_2016	RNA 3'-terminal phosphate cyclase	+	+	+	-	-	-	-
PCL1391_2021	Diaminopimelate decarboxylase	+	+	+	-	-	-	-
PCL1391_2037	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2051	RNA polymerase subunit sigma-70	+	+	+	+	-	+	-
PCL1391_2053	Putative TonB-dependent receptor	+	+	+	+	-	-	-
PCL1391_2076	Methyl-accepting chemotaxis protein	+	+	+	-	-	-	-
PCL1391_2141	Serralysin	+	+	+	-	-	-	-
PCL1391_2164	Alpha/beta hydrolase	+	+	+	-	+	+	-
PCL1391_2185	GNAT family acetyltransferase	+	+	+	+	+	-	-
PCL1391_2193	ABC transporter permease	+	+	+	-	+	+	-
PCL1391_2194	ABC transporter substrate-binding protein	+	+	+	-	+	+	-
PCL1391_2195	Methionine sulfoxide reductase A	+	+	+	-	-	-	-
PCL1391_2197	RND transporter	+	+	+	+	+	+	-
PCL1391_2199	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_2220	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2221	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2281	Acyl-CoA dehydrogenase	+	+	+	-	+	+	-
PCL1391_2405	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2433	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2479	Hypothetical protein	+	+	+	+	+	-	-
PCL1391_2481	Na/Pi cotransporter	+	+	+	-	-	-	-
PCL1391_2482	Hypothetical membrane protein	+	+	+	-	-	-	-
PCL1391_2483	Magnesium-transporting ATPase, P-type 1	+	+	+	+	+	+	-
PCL1391_2484	Conserved hypothetical protein	+	+	+	+	+	-	-
PCL1391_2556	MFS transporter	+	+	+	+	-	-	-
PCL1391_2557	L-2-hydroxyglutarate oxidase LhgO	+	+	+	+	-	-	-
PCL1391_2558	GntR family transcriptional regulator	+	+	+	+	-	-	-
PCL1391_2605	AraC family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_2609	Sulfite reductase	+	+	+	-	-	-	-
PCL1391_2610	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2645	Polyketide cyclase	+	+	+	+	+	+	-
PCL1391_2659	MFS transporter	+	+	+	+	-	+	-
PCL1391_2660	Oxidoreductase	+	+	+	-	-	-	-
PCL1391_2673	Transcriptional activator protein CzcR	+	+	+	+	+	+	-
PCL1391_2790	Glycosyltransferase	+	+	+	+	+	+	-
PCL1391_2887	Biopolymer transporter ExbD	+	+	+	+	+	-	-
PCL1391_2966	Non-hemolytic phospholipase C	+	+	+	-	-	-	-
PCL1391_2967	Membrane protein	+	+	+	-	-	-	-
PCL1391_2972	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_2973	Cyclic diguanylate phosphodiesterase	+	+	+	-	-	-	-
PCL1391_2987	Putative ABC transporter, permease subunit	+	+	+	+	+	-	-
PCL1391_2988	Putative ABC transporter, substrate-binding protein	+	+	+	+	+	-	-
PCL1391_2989	Putative ABC transporter, ATP-binding protein	+	+	+	+	+	-	-
PCL1391_2990	Acyl-CoA dehydrogenase	+	+	+	+	+	-	-

PCL1391_2992	AraC family transcriptional regulator	+	+	+	+	+	-	-
PCL1391_3032	Aminotransferase	+	+	+	-	+	+	-
PCL1391_3062	Amino acid transporter	+	+	+	-	-	-	-
PCL1391_3089	MFS transporter	+	+	+	-	-	-	-
PCL1391_3117	4-Hydroxyphenylacetate 3-monooxygenase oxygenase component	+	+	+	-	-	-	-
PCL1391_3126	(R,R)-Butanediol dehydrogenase	+	+	+	-	-	-	-
PCL1391_3130	Hypothetical protein	+	+	+	-	+	+	-
PCL1391_3144	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_3145	LysR family transcriptional regulator	+	+	+	+	+	+	-
PCL1391_3234	Transporter	+	+	+	-	-	-	-
PCL1391_3422	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_3423	Conserved hypothetical protein	+	+	+	+	+	+	-
PCL1391_3454	Response regulator FitH	+	+	+	-	-	-	-
PCL1391_3455	Transcriptional regulator FitG	+	+	+	-	-	-	-
PCL1391_3456	Sensor histidine kinase FitF	+	+	+	-	-	-	-
PCL1391_3457	Channel protein FitE	+	+	+	-	-	-	-
PCL1391_3458	Cytotoxin FitD	+	+	+	-	-	-	-
PCL1391_3459	Type I secretion system ATPase FitC	+	+	+	-	-	-	-
PCL1391_3479	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_3513	Putative glucosidase	+	+	+	+	+	+	-
PCL1391_3514	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_3515	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_3565	Molybdenum cofactor biosynthesis protein MoaA	+	+	+	-	-	-	-
PCL1391_3566	Molybdenum cofactor biosynthesis protein B	+	+	+	-	-	-	-
PCL1391_3569	Molybdopterin synthase catalytic subunit	+	+	+	-	-	-	-
PCL1391_3570	Molybdenum cofactor biosynthesis protein MoaD	+	+	+	-	-	-	-
PCL1391_3571	Molybdenum cofactor biosynthesis protein MoaC	+	+	+	-	-	-	-
PCL1391_3574	Molybdopterin-dependent oxidoreductase alpha subunit	+	+	+	-	-	-	-
PCL1391_3575	Cytochrome D ubiquinol oxidase subunit I	+	+	+	-	-	-	-
PCL1391_3576	Ubiquinol oxidase subunit II, cyanide insensitive	+	+	+	-	-	-	-
PCL1391_3600	TonB-denpendent receptor	+	+	+	+	+	+	-
PCL1391_3671	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_3751	Nucleoside 2-deoxyribosyltransferase	+	+	+	-	-	-	-
PCL1391_3843	Conserved hypothetical protein	+	+	+	+	+	-	-
PCL1391_3855	DNA polymerase subunit beta	+	+	+	+	+	+	-
PCL1391_3876	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_3932	HAD family hydrolase	+	+	+	-	-	-	-
PCL1391_3935	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_3937	Amidohydrolase	+	+	+	-	-	-	-
PCL1391_3989	Methyltransferase	+	+	+	-	-	-	-
PCL1391_4028	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_4037	Hemolysin secretion/activation protein, ShlB family	+	+	+	+	+	-	-
PCL1391_4083	LuxR family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_4176	Glutathione S-transferase	+	+	+	+	+	+	-
PCL1391_4307	Hypothetical protein	+	+	+	+	+	-	-
PCL1391_4350	AraC family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_4351	Fatty acid hydroxylase	+	+	+	-	-	-	-
PCL1391_4367	TetR family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_4386	Lysine transporter LysE	+	+	+	-	-	-	-
PCL1391_4387	LysR family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_4607	Putative arginase	+	+	+	+	-	+	-
PCL1391_4608	Transporter	+	+	+	+	-	+	-
PCL1391_4609	Transporter	+	+	+	+	-	+	-
PCL1391_4610	Fatty acid desaturase	+	+	+	-	-	-	-
PCL1391_4611	Structural protein MipA	+	+	+	+	+	+	-
PCL1391_4612	2,3-Diketo-5-methylthio-1-phosphopentane phosphatase	+	+	+	+	+	+	-
PCL1391_4613	Adenosylmethionine-8-amino-7-oxo-nanoate	+	+	+	+	+	+	-

	aminotransferase							
PCL1391_4614	Esterase	+	+	+	+	+	+	-
PCL1391_4615	Hypothetical protein	+	+	+	+	+	+	-
PCL1391_4616	Sensor histidine kinase	+	+	+	+	+	+	-
PCL1391_4617	Fis family transcriptional regulator	+	+	+	+	+	+	-
PCL1391_4626	50S ribosomal protein L31	+	+	+	+	+	-	-
PCL1391_4642	TonB-denpendent receptor	+	+	+	+	+	+	-
PCL1391_4646	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_4723	HIT family hydrolase	+	+	+	-	-	-	-
PCL1391_4735	Serine hydroxymethyltransferase	+	+	+	-	+	+	-
PCL1391_4798	Hypothetical membrane protein	+	+	+	+	-	-	-
PCL1391_4800	XRE family transcriptional regulator	+	+	+	+	-	-	-
PCL1391_4801	Histidine kinase	+	+	+	+	-	-	-
PCL1391_4891	GNAT family acetyltransferase	+	+	+	-	-	-	-
PCL1391_4896	D-alanyl-alanine synthetase	+	+	+	-	+	+	-
PCL1391_4904	MFS transporter	+	+	+	-	-	-	-
PCL1391_4905	LysR family transcriptional regulator	+	+	+	-	-	-	-
PCL1391_4907	Short-chain dehydrogenase	+	+	+	+	-	-	-
PCL1391_4917	Cobalt-zinc-cadmium resistance protein CzcD	+	+	+	+	+	-	-
PCL1391_4925	Acid phosphatase	+	+	+	+	+	+	-
PCL1391_4982	Hypothetical protein	+	+	+	-	+	+	-
PCL1391_4983	Glycosyl transferase PslA	+	+	+	+	+	+	-
PCL1391_4985	Glycosyl transferase PslC	+	+	+	+	+	+	-
PCL1391_4986	Polysaccharide biosynthesis/export protein PslD	+	+	+	+	+	+	-
PCL1391_4987	Polysaccharide biosynthesis/export protein PslE	+	+	+	+	+	+	-
PCL1391_4988	glycosyl transferase PslF	+	+	+	+	+	+	-
PCL1391_4989	Glycosyl hydrolase PslG	+	+	+	+	+	+	-
PCL1391_4990	Glycosyl transferase PslH	+	+	+	+	+	+	-
PCL1391_4991	Glycosyl transferase PslI	+	+	+	+	+	+	-
PCL1391_4992	Membrane protein PslJ	+	+	+	+	+	+	-
PCL1391_4993	Acetyltransferase	+	+	+	+	-	-	-
PCL1391_4994	Membrane protein PslK	+	+	+	+	+	+	-
PCL1391_5052	Hypothetical protein	+	+	+	-	+	+	-
PCL1391_5077	Benzoate transporter	+	+	+	+	-	+	-
PCL1391_5099	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_5179	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_5182	Copper-containing nitrite reductase	+	+	+	-	-	-	-
PCL1391_5360	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_5397	Aminoglycoside N(6')-acetyltransferase	+	+	+	-	-	-	-
PCL1391_5511	Putative membrane protein	+	+	+	-	-	-	-
PCL1391_5577	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate ami-transferase	+	+	+	+	+	+	-
PCL1391_5659	Membrane protein	+	+	+	-	+	+	-
PCL1391_5765	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_5770	Hypothetical protein	+	+	+	-	-	-	-
PCL1391_5773	Membrane protein	+	+	+	+	+	+	-
PCL1391_5799	TraR family zinc finger protein	+	+	+	+	+	-	-
PCL1391_5806	Phosphoribosyl-AMP cyclohydrolase 2	+	+	+	-	+	+	-

+, gene present; -, gene absent

Loci shaded in grey are discussed in the text.

References

Auch A. F., von Jan M., Klenk H.-P., Goeker M. (2010). Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* 2**:** 117-134.

Blom J., Albaum S. P., Doppmeier D., Pühler A., Vorhölter F.-J., Zakrzewski M. *et al.* (2009). EDGAR: a software framework for the comparative analysis of prokaryotic genomes. *BMC Bioinformatics* 10**:** 154.

Boetzer M., Henkel C. V., Jansen H. J., Butler D., Pirovano W. (2011). Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics* 27**:** 578-579.

Boetzer M., Pirovano W. (2012). Toward almost closed genomes with GapFiller. *Genome Biol* 13**:** R56.

Burr S. E., Gobeli S., Kuhnert P., Goldschmidt-Clermont E., Frey J. (2010). *Pseudomonas chlororaphis* subsp. *piscium* subsp. nov., isolated from freshwater fish. *Int J Syst Evol Microbiol* 60**:** 2753-2757.

Chin-A-Woeng T. F. C., Bloemberg G. V., van der Bij A. J., van der Drift K., Schripsema J., Kroon B. *et al.* (1998). Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant-Microbe Interact* 11**:** 1069-1077.

Gomila M., Peña A., Mulet M., Lalucat J., García-Valdés E. (2015). Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 6**:** 214.

Gupta G. P., Rani S., Birah A., Raghuraman M. (2005). Improved artificial diet for mass rearing of the tobacco caterpillar, *Spodoptera litura* (Lepidoptera: Noctuidae). *Int J Trop Insect Sci* 25**:** 55-58.

Jousset A., Schuldes J., Keel C., Maurhofer M., Daniel R., Scheu S. *et al.* (2014). Full-genome sequence of the plant growth-promoting bacterium *Pseudomonas protegens* CHA0. *Genome Announc* 2**:** e00322-00314.

Keel C., Weller D. M., Natsch A., Defago G., Cook R. J., Thomashow L. S. (1996). Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl Environ Microbiol* 62**:** 552-563.

King E. O., Ward M. K., Raney D. E. (1954). 2 simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* 44**:** 301-307.

Konstantinidis K. T., Ramette A., Tiedje J. M. (2006). The bacterial species definition in the genomic era. *Phil Trans R Soc B* 361**:** 1929-1940.

Martinez-Garcia E., de Lorenzo V. (2011). Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Environ Microbiol* 13**:** 2702-2716.

Meier-Kolthoff J. P., Auch A. F., Klenk H. P., Goker M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14**:** 60.

Meier-Kolthoff J. P., Klenk H.-P., Göker M. (2014). Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. *Int J Syst Evol Microbiol* 64**:** 352-356.

Mulet M., Gomila M., Scotta C., Sánchez D., Lalucat J., García-Valdéz E. (2012). Concordance between whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry and multilocus sequence analysis approaches in species discrimination within the genus *Pseudomonas*. *Syst Appl Microbiol* 35**:** 455-464.

Peix A., Valverde A., Rivas R., Igual J. M., Ramírez-Bahena M.-H., Mateos P. F. *et al.* (2007). Reclassification of *Pseudomonas aurantiaca* as a synonym of *Pseudomonas chlororaphis* and proposal of three subspecies, *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. *Int J Syst Evol Microbiol* 57**:** 1286-1290.

Perneel M., Heyrman J., Adiobo A., De Maeyer K., Raaijmakers J. M., De Vos P. *et al.* (2007). Characterization of CMR5c and CMR12a, novel fluorescent *Pseudomonas* strains from the cocoyam rhizosphere with biocontrol activity. *J Appl Microbiol* 103**:** 1007-1020.

Ramette A., Frapolli M., Fischer-Le Saux M., Gruffaz C., Meyer J.-M., Défago G. *et al.* (2011). *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. *Int J Syst Evol Microbiol* 34**:** 180-188.

Rezzonico F., Zala M., Keel C., Duffy B., Moenne-Loccoz Y., Defago G. (2007). Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? *New Phytol* 173**:** 861-872.

Ruffner B., Péchy-Tarr M., Ryffel F., Hoegger P., Obrist C., Rindlisbacher A. *et al.* (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ Microbiol* 15**:** 751-763.

Ruffner B., Péchy-Tarr M., Höfte M., Bloemberg G., Grunder J., Keel C. *et al.* (2015). Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* 16**:** 609

Sambrook J., Russel D. W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: New York.

Sharifi-Tehrani A., Zala M., Natsch A., Moenne-Loccoz Y., Defago G. (1998). Biocontrol of soil-borne fungal plant diseases by 2,4-diacetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. *Eur J Plant Pathol* 104**:** 631-643.

Stutz E. W., Défago G., Kern H. (1986). Naturally-occuring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* 76**:** 181-185.

Tambong J. T., Höfte M. (2001). Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. *Eur J Plant Pathol* 107**:** 511-521.

Venables W. N., Ripley B. D. (2002). *Modern Applied Statistics with S*, Fourth edn. Springer: New York.

CHAPTER 3

Antimicrobial and insecticidal: cyclic lipopeptides and hydrogen cyanide produced by plant-beneficial *Pseudomonas* strains CHA0, CMR12a and PCL1391 contribute to insect killing

This chapter was submitted to *Frontiers in Microbiology* where it was accepted with minor revisions (19th Oct 2016)

Pascale Flury[1], Pilar Vesga[1], Maria Péchy-Tarr[2], Nora Aellen[1], Francesca Dennert[1], Nicolas Hofer[1], Karent Paola Kupferschmied[2], Peter Kupferschmied[2], Zane Metla[1,3], Zongwang Ma[4], Sandra Siegfried[1], Sandra de Weert[5, 6], Guido Bloemberg[5,7], Monica Höfte[4], Christoph Keel[2]* and Monika Maurhofer[1]*

[1]Plant Pathology, Institute of Integrative Biology, ETH Zürich, Zürich, Switzerland
[2]Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland
[3]Laboratory of Experimental Entomology, Institute of Biology, University of Latvia, Latvia
[4]Laboratory of Phytopathology, Faculty of Bioscience Engineering, Ghent University, B-9000 Ghent, Belgium
[5]Institute of Biology Leiden, Leiden University, The Netherlands
[6]Present address: Koppert, Berkel en Rodenrijs, The Netherlands
[7]Present address: Unilabs, Dübendorf, Switzerland
*corresponding authors

ABSTRACT

Particular groups of plant-beneficial fluorescent pseudomonads are not only root colonizers that provide plant disease suppression, but in addition are able to infect and kill insect larvae. The mechanisms by which the bacteria manage to infest this alternative host, to overcome its immune system, and to ultimately kill the insect is still largely unknown. However, the investigation of the few virulence factors discovered so far, points to a highly multifactorial nature of insecticidal activity. Antimicrobial compounds produced by fluorescent pseudomonads are effective weapons against a vast diversity of organisms such as fungi, oomycetes, nematodes and protozoa. Here, we investigated whether these compounds also contribute to insecticidal activity. We tested mutants of the highly insecticidal strains *Pseudomonas protegens* CHA0, *Pseudomonas chlororaphis* PCL1391, and *Pseudomonas* sp. CMR12a, defective for individual or multiple antimicrobial compounds, for injectable and oral activity against lepidopteran insect larvae. Moreover, we studied expression of biosynthesis genes for these antimicrobial compounds for the first time in insects. Our survey revealed that hydrogen cyanide and different types of cyclic lipopeptides contribute to insecticidal activity. Hydrogen cyanide was essential to full virulence of CHA0 and PCL1391 directly injected into the hemolymph. The cyclic lipopeptide orfamide produced by CHA0 and CMR12a was mainly important in oral infections. Mutants of CMR12a and PCL1391 impaired in the production of the cyclic lipopeptides sessilin and clp1391, respectively, showed reduced virulence in injection and feeding experiments. Although virulence of mutants lacking one or several of the other antimicrobial compounds, i.e. 2,4-diacetylphloroglucinol, phenazines, pyrrolnitrin, or pyoluteorin, was not reduced, these metabolites might still play a role in an insect background since all investigated biosynthetic genes for antimicrobial compounds of strain CHA0 were expressed at some point during insect infection. In summary, our study identified new factors contributing to insecticidal activity and extends the diverse functions of antimicrobial compounds produced by fluorescent pseudomonads from the plant environment to the insect host.

INTRODUCTION

Root-colonizing fluorescent pseudomonads are well known for their plant-beneficial traits, which include inhibition of root-pathogens, induction of resistance in the plant and solubilization of mineral nutrients. Nevertheless, during the last decade evidence arose that we should widen our view on their life-style since plant roots apparently are not the only environment colonized by these bacteria. Indeed, many strains throughout the *Pseudomonas fluorescens* group were discovered to have the ability to colonize insects and strains of two sub-clades can even cause lethal infections (Péchy-Tarr *et al.*, 2008; Olcott *et al.*, 2010; Ruffner *et al.*, 2013; Ruffner *et al.*, 2015; Flury *et al.*, 2016; Keel, 2016). Especially strains of sub-clade 1 (Loper *et al.*, 2012), also called the *Pseudomonas chlororaphis* subgroup (Gomila *et al.*, 2015), are highly insecticidal when injected, but also when ingested by insect larvae (Ruffner *et al.*, 2015; Flury *et al.*, 2016). How exactly plant-associated pseudomonads colonize insects and which factors are decisive for fatal infections is still largely unknown.

To date the most intensively studied virulence factor against insects is the Fit toxin, which is similar to Mcf1 of the entomopathogenic bacterium *Photorhabdus luminescens* (Péchy-Tarr *et al.*, 2008; Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014). Fit toxin-negative mutants of *Pseudomonas protegens* strains CHA0 and Pf-5 exhibited reduced toxicity when injected into the hemolymph of *Galleria mellonella* or *Manduca sexta* larvae (Péchy-Tarr *et al.*, 2008). Fit mutants of *P. protegens* CHA0 and *P. chlororaphis* PCL1391 further showed reduced virulence when fed to larvae of *Spodoptera littoralis* (Ruffner *et al.*, 2013). However, all Fit mutants retained significant virulence, and in oral infections of *Drosophila melanogaster* by *P. protegens* Pf-5 no role for the Fit toxin could be detected (Loper *et al.*, 2016). This indicates the involvement of additional virulence factors and points to a certain specificity of virulence factors to individual insect species (Keel, 2016).

For oral insecticidal activity a functional GacS/GacA-regulatory system is essential (Ruffner *et al.*, 2013; Loper *et al.*, 2016). Accordingly, a Fit-GacA double mutant was strongly reduced in virulence (Ruffner *et al.*, 2013) in oral infections of *S. littoralis*. Recently, chitinase C was identified as one of the Gac-regulated factors contributing to toxicity of *P. protegens* CHA0 and Pf-5 towards *Plutella xylostella* and *D. melanogaster*, respectively (Flury *et al.*, 2016; Loper *et al.*, 2016). While some factors, such as the Fit

toxin or chitinase C, are present throughout all strains of the *P. chlororaphis* subgroup, the observation of strain-specific differences in toxicity further suggested the existence of strain-specific factors (Flury *et al.*, 2016). Rhizoxin seems to be such a factor as it strongly contributes to insecticidal activity of *P. protegens* Pf-5, but is not produced by most other strains belonging to the *P. chlororaphis* subgroup (Loper *et al.*, 2016). Generally, antimicrobial compounds produced by strains of the *P. chlororaphis* subgroup, such as 2,4-diacetylphloroglucinol (Phl), pyrrolnitrin (Prn), pyoluteorin (Plt), hydrogen cyanide (Hcn), phenazines (Phz) and cyclic lipopeptides (Clp) represent possible candidates for a role in insecticidal activity. These compounds exhibit toxic effects towards a broad spectrum of organisms (Gross and Loper, 2009) and their production is Gac-regulated (Hassan *et al.*, 2010; Kidarsa *et al.*, 2013). Besides their well-demonstrated contribution to biocontrol activity of pseudomonads against fungal pathogens, also activity against bacteria, protists, nematodes, arthropods, plants and mammalian cells is reported (Keel *et al.*, 1992; Maurhofer *et al.*, 1992; Maurhofer *et al.*, 1995; Devi and Kothamasi, 2009; Gross and Loper, 2009; Neidig *et al.*, 2011; Nisr *et al.*, 2011; Jang *et al.*, 2013).

The aim of this study was to investigate whether antimicrobial compounds are also important for these pseudomonads when infecting an insect host. The highly insecticidal *P. chlororaphis* subgroup includes three species*: P. protegens*, *P. chlororaphis* and a yet to be named species comprising strains such as *Pseudomonas* sp. CMR12a and *Pseudomonas* sp. CMR5c (Flury *et al.*, 2016). Since the different species produce distinct sets of antimicrobial compounds we selected one representative strain per species: *P. protegens* CHA0, *P. chlororaphis* PCL1391 and *Pseudomonas* sp. CMR12a. All three strains produce Hcn and *P. protegens* CHA0 additionally produces Phl, Plt and Prn while *P. chlororaphis* PCL1391 and *Pseudomonas* sp. CMR12 produce phenazine-1-carboxylic acid and phenazine-1-carboxamide (Chin-A-Woeng *et al.*, 1998; Haas and Keel, 2003; Perneel *et al.*, 2007). CMR12a further produces two Clps, orfamide (Ofa) and sessilin (Ses) (D'aes *et al.*, 2011; D'aes *et al.*, 2014), the latter of which is closely related to tolaasin of the mushroom pathogen *Pseudomonas tolaasii* (Bassarello *et al.*, 2004). Recently, orfamide production was also demonstrated for *P. protegens* CHA0 (Ma *et al.*, 2016) and *P. chlororaphis* PCL1391 was found to harbor genes for the synthesis of a Clp (Flury *et al.*, 2016). Here, we screened newly generated as well as existing mutants deficient for one or several of the different antimicrobial metabolites for their toxic

activity towards insects either when injected directly into the hemocoel or when taken up orally. Furthermore, virulence of mutants deficient for additional compounds or enzymes contributing to biocontrol, i.e. pyoverdine (Pvd), extracellular protease AprA (AprA), glucose dehydrogenase (Gcd) and gluconate dehydrogenase (Gad) (Keel *et al.*, 1989; Siddiqui *et al.*, 2005; de Werra *et al.*, 2009), that potentially could give an advantage during insect colonization, was investigated. Finally, expression of biosynthetic genes for a selection of the investigated metabolites was studied in CHA0 during the process of insect infection.

Our findings highlight major contributions to insecticidal activity for all investigated Clps as well as for Hcn, but not for any of the other metabolites or enzymes.

MATERIALS AND METHODS

Bacterial growing conditions

The bacterial strains used in this study are listed in Table 1. For all insect assays bacterial strains taken from our long-term strain storage at -80°C were grown for two days at 24°C on King's medium B agar (King *et al.*, 1954) supplemented with ampicillin (40 µg ml^{-1}), chloramphenicol (13 µg ml^{-1}), and cycloheximide (100 µg ml^{-1}) or supplemented with kanamycin (50 µg ml^{-1}) depending on the strain. These cultures were used to inoculate 10 ml LB medium (Bertani, 1951). Bacterial cultures were incubated overnight with rotational shaking (180 rpm) at 24°C. Cells were washed twice or three times in sterile 0.9% NaCl solution for feeding or injection experiments, respectively, and diluted to the required concentration.

Construction of deletion mutants of *P. protegens* CHA0 and *P. chlororaphis* PCL1391

Mutants of *P. protegens* CHA0 and *P. chlororaphis* PCL1391 with deletions in the biosynthetic genes for antimicrobial compounds were created by means of an allelic replacement technique using the I-SceI system with the suicide vector pEMG (Martinez-Garcia and de Lorenzo, 2011) as described by Kupferschmied *et al.* (2014). Briefly, using the primer pairs listed in Supplementary Table S1, the 600-700-bp upstream and downstream regions flanking the genomic region to be deleted were amplified by PCR. After digestion with the indicated restriction enzymes (Supplementary Table S1), the fragments were cloned into pEMG via triple ligation yielding the final suicide plasmid which was verified by sequencing. Next, using the I-SceI system with the expression plasmid pSW-2, the deletion mutants CHA1241, CHA5091, CHA5092, CHA5101, CHA5103, CHA5098, CHA5118 and PCL5103 were generated (Table 1).

Table 1 Strains used in this study

Strain	Genotype or phenotype	Defective in the production of	Reference or comment
Pseudomonas protegens			
CHA0	Wild type, isolated from tobacco roots		Stutz *et al.* (1986); Jousset *et al.* (2014)
CHA19	Δ*gacS* deletion mutant of CHA0	GacS sensor	Zuber *et al.* (2003)
CHA89	*gacA*::Km^r insertion mutant of CHA0	GacA response regulator	Laville *et al.* (1992)
CHA400	*pvd-400*::Tn*1733* insertion mutant of CHA0; Km^r	Pyoverdine (Pvd)	Keel *et al.* (1989)
CHA805	Nonpolar *aprA*::*'lacZ* insertion mutant of CHA0	Protease AprA (AprA)	Siddiqui *et al.* (2005)
CHA805-g	CHA805::attTn*7-gfp2* , Gm^r	AprA	This study
CHA1151	Δ*fitD* in-frame deletion mutant of CHA0	Fit toxin (Fit)	Péchy-Tarr *et al.* (2008)
CHA1196	Δ*gcd* in-frame deletion mutant of CHA0	Glucose dehydrogenase (Gcd)	de Werra *et al.* (2009)
CHA1197	Δ*gad* in-frame deletion mutant of CHA0	Gluconate dehydrogenase (Gad)	de Werra *et al.* (2009)
CHA1241	Δ*phlACBD* deletion mutant of CHA0	2,4-Diacetylphloroglucinol (Phl)	This study
CHA1281	Δ*fitD gacA*::Km^r mutant of CHA0	Fit toxin and GacA	Ruffner *et al.* (2013)
CHA5091	Δ*prnABCD* deletion mutant of CHA0	Pyrrolnitrin (Prn)	This study
CHA5092	Δ*pltABCDEFG* deletion mutant of CHA0	Pyoluteorin (Plt)	This study
CHA5098	Δ*phlACBD* Δ*prnABCD* Δ*pltABCDEFG* deletion mutant of CHA0	Phl/Prn/Plt	This study
CHA5101	Δ*ofaABC* deletion mutant of CHA0	Orfamide (Ofa)	This study
CHA5103	Δ*hcnABC* deletion mutant of CHA0	Hydrogen cyanide (Hcn)	This study
CHA5118	Δ*phlACBD* Δ*prnABCD* Δ*pltABCDEFG* - Δ*hcnABC* Δ*ofaABC* deletion mutant of CHA0	Phl/Prn/Plt/Hcn/Ofa	This study
Pseudomonas chlororaphis			
PCL1391	Wild type, isolated from tomato roots		Chin-A-Woeng *et al.* (1998)
PCL1113	*phzF*:: Tn*5-luxAB* insertion mutant of PCL1391homolog; Km^r	Phenazine-1-carboxylic acid and phenazine-1-carboxamide (Phz)	Chin-A-Woeng *et al.* (1998)
PCL1123	*gacS*::Tn*5-luxAB* insertion mutant of PCL1391; Km^r	GacS	This study
PCL1832	*PCL1391_3318*::Tn5-luxAB insertion mutant of PCL1391; Km^r	Clp1391	This study
PCL5103	Δ*hcnABC* deletion mutant of PCL1391	Hcn	This study

Table 1 continued

Strain	Genotype or phenotype	Defective in the production of	Reference or comment
Pseudomonas sp.			
CMR12a	Wild type, isolated from cocoyam roots		Perneel *et al.* (2007)
CMR12a-ΔPhz	Δ*phzABCDEFGH* deletion mutant of CMR12a	Phenazine (Phz)	D'aes *et al.* (2011)
CMR12a-Clp1	*sesA* insertion mutant, Gm^r	Sessilin (Ses)	D'aes *et al.* (2011)
CMR12a-ΔPhz-Clp1	Δ*phzABCDEFGH sesA* mutant of CMR12, Gm^r	Phz/Ses	D'aes *et al.* (2011)
CMR12a-ΔClp2	Δ*ofaBC* deletion mutant of CMR12a	Orfamide (Ofa)	D'aes *et al.* (2014)
CMR12a-ΔPhz-ΔClp2	Δ*phzABCDEFGH* Δ*ofaBC* mutant of CMR12a	Phz/Ofa	D'aes *et al.* (2014)
CMR12a-ΔClp2-Clp1	Δ*ofaBC sesA* mutant of CMR12a, Gm^r	Ofa/Ses	D'aes *et al.* (2014)
CMR12a-ΔPhz-ΔClp2-Clp1	Δ*phzABCDEFGH* Δ*ofaBC sesA* mutant of CMR12a, Gm^r	Phz/Ofa/Ses	D'aes *et al.* (2014)
Escherichia coli			
DH5α	*recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)U169(Φ80d*lacZ*ΔM15)		Sambrook and Russel (2001)
S17-1/λpir	*pro thi hsdR recA chromosome*::RP4-2; Tc::Mu Km::Tn*7*/ λpir; Tp^r Sm^r		Simon *et al.* (1983)

Gm^r, gentamicin; and Km^r, kanamycin resistance, respectively.

Transposon mutagenesis of PCL1391

Additional mutants of *P. chlororaphis* PCL1391 were generated by random transposon mutagenesis. Plasmid pRL1063A (Wolk *et al.*, 1991), which harbors the Tn5luxAB transposon and a kanamycin marker gene, was used to generate transconjugants of PCL1391. Nalidixic acid was added to a final concentration of 15 µg/ml to reduce growth of the *E. coli* helper strains, carrying pRL1063A and pRK2013 (Ditta *et al.*, 1980). A library of Tn5luxAB mutants of PCL1391 was screened for loss of drop collapsing activity, which resulted in the selection of biosurfactant mutants. To recover the regions flanking the transposon insertion site, chromosomal DNA of the mutant was isolated and digested with EcoRI. After re-circulation and transformation into *E. coli* DH5α, the Tn5luxAB flanking chromosomal DNA regions were sequenced. The primers used, i.e. oMP407 (5'TACTAGATTCAATGCTATCAATGAG-3') and oMP408 (5'-AGGAGGTCACATGGAATATCAGAT-3'), were homologous to the left and right border of

the Tn5luxAB, respectively. Sequences obtained were analyzed using BLASTX in GENbank (Altschul *et al.*, 1997). Sequence analysis of the Tn5luxAB flanking regions revealed that for one mutant, strain PCL1123, the transposon was inserted at bp 2575/2576 in the global regulatory gene for secondary metabolites, *gacS* (Workentine *et al.*, 2009). For a second mutant (referred to as PCL1832) the transposon was inserted in locus PCL1391_3318 at bp 9621/9622. PCL1391_3318 is homologous (>70% nucleotide sequence identity) to the orfamide biosynthetic gene *ofaC* of *P. protegens* Pf-5 (Gross *et al.*, 2007).

Hcn production assay

To test for the production of Hcn, bacteria were grown on King's medium B agar. An Hcn-indicator paper (Whatman 3M, soaked in a solution of Cu (II) ethylacetoacetate (5 mg; Kodak) + 4.4'-methylene-bis-N,N-dimethyl-aniline (5 mg; Fluka) per ml chloroform, dried and stored in the dark (Castric and Castric, 1983)) was placed in the lid of the petri-dish. After incubation at 24°C for 24 h blue coloration indicated Hcn production (Voisard *et al.*, 1989).

Droplet collapse assay

To test whether bacterial strains were able to decrease the surface tension, the ability to collapse a droplet of water on a Parafilm "M" laboratory film (American National Can, Chicago, IL) was assessed (Jain *et al.*, 1991). 25 or 50 µl King's medium B culture supernatant was pipetted directly onto Parafilm. The flattening and spreading of the droplet (drop collapsing activity) was used as an indicator of biosurfactant production.

Swarming assay

Surface swarming motility was assessed on soft agar LB plates (0.6% w/v agar). *Pseudomonas* strains were grown in King's medium B with rotational shaking (150 rpm) at 28ºC for 24 h. Bacterial cells were washed three times in sterile water and 5 µl of cell suspensions were pipetted onto the center of the plates. For complementation of the Ofa$^-$ mutant CHA5101, orfamide A of *P. protegens* CHA0, which was purified as described by Ma *et al.* (2016), was added to the medium. Pictures were taken after 20 h of incubation at 28°C.

Galleria injection assay

Injection assays with *G. mellonella* were performed as described by Flury *et al.* (2016) with small adaptations. In each repetition of the experiment three times ten last-instar *Galleria mellonella* larvae (Hebeisen Fishing, Zürich, Switzerland) were injected with $2x10^3$ washed bacterial cells suspended in 10 µl of 0.9% sterile NaCl using a repetitive dispensing Tridak Stepper (Intertronic, Oxfordshire, UK). Injected suspensions were further plated in three dilutions on King's medium B agar to confirm that suspensions of different strains indeed contained the same number of bacteria. Larvae were kept in Petri dishes at 24°C in the dark. When they started to become melanized they were scored as live or dead every hour. Every mutant strain was tested at least three times. To exclude a human bias, the experiments were performed double-blind, i.e. the set-up of the experiments and the scoring of the larvae was performed without knowledge of the treatments.

***Plutella* feeding assays**

Feeding assays were performed as described by Flury *et al.* (2016). Eggs of *P. xylostella* were obtained from Syngenta Crop Protection AG (Stein, Switzerland). General growth conditions for larvae before and during the experiments were 26°C, 60% humidity and a 16-h day, 8-h night cycle. 1-week-old larvae (deriving from four different egg batches) were used for experiments during which each larva was kept separately in 128-cell bioassay trays (Frontier Agricultural Sciences, Delaware, USA) to prevent injuries due to cannibalism. The bottom of each well of the bioassay trays was covered by a wetted filter paper on which a pellet of insect diet was placed and inoculated with 10 µl of a suspension of washed bacterial cells. For insect diet preparation 500 ml of sterile ddH_2O containing 7.5 g Agar was boiled for 1 min. Next, 50 g Adapta Bio-Dinkel (Hero Baby, Switzerland), one effervescent vitamin pill Santogen Gold (Coop, Switzerland), 15.5 g yeast extract, 7.5 g casamino acids, 0.25 g cholesterol and 0.5 ml corn oil were added and all ingredients were mixed thoroughly with a blender. The hot mixture was poured into Petri dishes to a height of approximately 1.5 mm. From the solidified insect diet food pellets were cut with a sterile cork borer (4 mm diameter). For *P. protegens* CHA0 and its mutant derivatives, the experiments were performed with a bacterial dose of 4 x 10^6 cfu/ pellet and a total of 64 larvae per treatment. For *P. chlororaphis* PCL1391, *Pseudomonas* sp. CMR12a and derivatives, a dose of 2 x 10^7 cfu/pellet was used and 32

larvae were tested for each treatment. Larvae were scored as live or dead regularly over time. Every mutant strain was tested at least three times. To exclude a human bias the experiments were performed double-blind as described above.

Gene expression analysis

To study bacterial gene expression in the insect host, *G. mellonella* and *P. xylostella* were infected with *P. protegens* CHA0 as described above. For experiments with *G. mellonella*, gene expression was studied 20 h, 30 h and 42 h post-injection. At 42 h infected larvae had died while control larvae were still alive. Hemolymph was collected from a cut near the tail of surface disinfested larvae (20s in 70% Ethanol, rinsed in sterile ddH_2O) and frozen in liquid nitrogen. *P. xylostella* larvae were collected after 20 h, 30 h, and as soon as death occurred. Larvae were washed in ddH_2O, homogenized and frozen in liquid nitrogen. RNA was extracted from both insect samples using the Trizol-Reagent protocol ™ (Thermo Fisher Scientific, Massachusetts, USA) and quantified with a NanoDrop™ 2000 (Thermo Fisher Scientific, Massachusetts, USA). In order to verify the absence of contaminating genomic DNA, a PCR was performed with primers specific for the *16S rRNA* gene of *Pseudomonas* spp. (Bergmark *et al.*, 2012). For each sample within one experiment, equal amounts of RNA were then converted into cDNA using the GoScript™ Reverse Transcription System (Promega, Wisconsin, USA) according to the instructions of the manufacturer. The cDNA was amplified by PCR with Thermo Scientific™ DreamTaq™ DNA Polymerase (Thermo Fisher Scientific) using the primers listed in Supplementary Table S2. The presence of the amplicon was verified by electrophoresis in1.5% or 3% agarose gels, depending on the expected size of the PCR product. The gel was post-stained with GelRed™ (Invitrogen, California, USA) and the bands visualized with the ChemiDoc™ XRS+ System (BioRad, California, USA).

Statistics

Data analysis was performed in RStudio version 0.98.1017 (http://www.rstudio.com). To test for significant differences between survival curves of wild-type and mutant strains, the Log-Rank test of the Survival package of R was applied. LT_{50} values were calculated using a generalized linear model in the MASS package.

RESULTS

Hydrogen cyanide mainly contributes to injectable insect toxicity

In frame deletion mutants for hydrogen cyanide were generated for strains *P. protegens* CHA0 and *P. chlororaphis* PCL1391. Both mutants were no longer able to produce the HCN as confirmed with an Hcn-indicator paper assay (data not shown). When injected into the hemocoel of *G. mellonella* larvae, Hcn-deficient mutants of both strains killed the larvae significantly slower than the respective wild type according to a Log-Rank test (Figure 1A and B) and significantly higher LT_{50} values (Table 2). A similar trend was

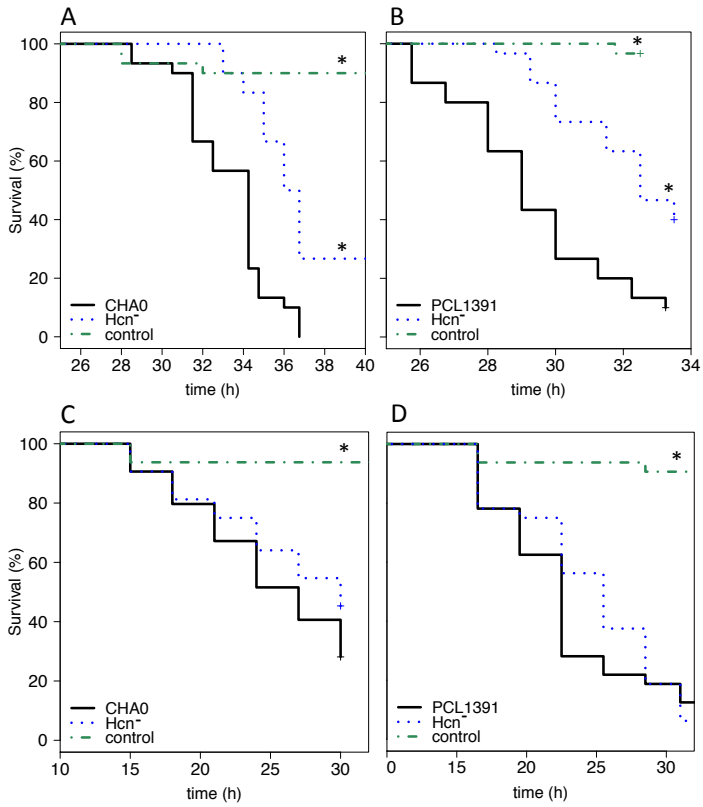


Figure 1 Hydrogen cyanide-deficient mutants exhibit reduced injectable insecticidal activity. (A, B) Injectable activity against *Galleria mellonella*. Thirty larvae per treatment were injected with 2 x 10^3 bacterial cells and survival was recorded hourly. **(C, D)** Oral activity against *Plutella xylostella*. For *Pseudomonas protegens* CHA0 **(C)** 64 larvae were exposed to artificial diet inoculated with 4 x 10^6 bacterial cells and for *Pseudomonas chlororaphis* PCL1391 **(D)** 32 larvae were exposed to 2 x 10^7 bacterial cells. Sterile 0.9% NaCl solution served as control. Treatments that differed significantly from their respective wild-type strain (Log-Rank test $p \leq 0.05$, Survival Package in R) are marked with an asterisk. LT_{50} values of the experiments presented above and of a repetition of the experiment are listed in Tables 2 and 3. Each strain was tested at least three times and similar results were obtained. Solid black line, wild-type strain; dotted blue line, mutants deficient for hydrogen cyanide production (Hcn^-, CHA5103, PCL5103); dash-dot green line, 0.9% NaCl solution control.

observed in feeding assays with *P. xylostella*. In two out of three experiments, the Hcn deficient mutant of CHA0 showed significantly higher LT_{50} values than the wild type (Table 3). However, the Log-Rank test between CHA0 and its Hcn-negative mutant CHA5103 was only nearly significant (p=0.06) (Figure 1C) and for PCL1391 only a tendency for a longer kill-time, but no significant effects were found in oral infections (Figure 1D). Hence, the lack of Hcn seems to have a pronounced impact on injectable insecticidal activity, but rather a minor effect in oral infections.

Different cyclic lipopeptides all contribute to insect toxicity

The impact of Clps on insecticidal activity was studied in all three *Pseudomonas* strains. Clp single and double mutants of CMR12a were already available (D'aes *et al.*, 2011; D'aes *et al.*, 2014). Here, a mutant of CHA0 deficient for all three peptide synthases required for the production of orfamide A (Ma *et al.*, 2016) was generated and termed CHA5101. This mutant neither reduced surface tension as indicated by a droplet collapse test nor showed swarming motility on soft agar plates (Supplementary Figure S1A, B). However, swarming ability was regained when plates were supplemented with orfamide A (Supplementary Figure S1A). Further, a Clp biosynthesis-defective mutant of PCL1391 with a Tn5 insertion in an *ofaC* homolog (PCL1832) was created and found to be impaired in causing droplet collapse (Supplementary Figure S1C). The Clp of PCL1391 will be called Clp1391 in the following. A detailed characterization of Clp1391 and the mutant PCL1832 will be presented elsewhere.

When injected into the hemocoel of *G. mellonella* larvae the Ofa^- mutants of CHA0 and CMR12a did not behave differently from the respective wild-type strains (Figure 2A, C and Table 2). In contrast, the $Clp1391^-$ mutant of PCL1391 killed always significantly slower than the wild parent strain, though, still causing 100% mortality at the end of the experiment (Figure 2B, Table 2). A clear reduction in virulence was also observed for the Ses^- mutant of CMR12a represented by significantly higher LT_{50} values in all repetitions of the experiment and also a lower killing rate (Figure 2C, Table 2).

Table 2 Lethal time 50 (LT_{50}) for *Galleria mellonella* larvae injected with wild type and mutant *Pseudomonas* strains

Strain	Phenotype	LT_{50} (h)			
		Experiment 1	Experiment 2	Experiment 3	Experiment 4
CHA0	Wild type	29.1 (28.8; 29.4)	32.9 (32.5; 33.3)	33.8 (33.4; 34.1)	33.7 (33.3; 34.1)
CHA1151	Fit$^-$	30.6 (30.1; 31.1)*	32.8 (32.5; 33.2)	34.6 (34.2; 35.0)*	36.3 (35.8; 36.7)*
CHA1281	GacA$^-$Fit$^-$	31.8 (31.3; 32.2)*	35.3 (34.6; 35.9)*	34.1 (33.7; 34.5)	36.8 (36.3; 37.3)*
CHA400	Pvd$^-$			33.0 (32.4; 33.6)	34.1 (33.6; 34.5)
CHA1196	Gcd$^-$			34.7 (34.0; 34.8)	34.7 (34.2; 35.2)*
CHA1197	Gad$^-$			33.9 (33.4; 34.3)	30.4 (29.8; 30.9)†
CHA805-g	AprA$^-$			33.6 (33.3; 34.0)	32.7 (32.3; 33.0)†
CHA1241	Phl$^-$	28.7 (28.3; 29.1)	33.0 (32.6; 33.5)		
CHA5091	Prn$^-$	28.5 (28.2; 28.9)	32.7 (32.3; 33.0)		
CHA5092	Plt$^-$	28.6 (28.3; 29.0)	30.8 (30.3; 31.3)†		
CHA5101	Ofa$^-$	28.2 (27.6; 28.8)	30.9 (30.3; 31.4)†		
CHA5103	Hcn$^-$	30.9 (30.5; 31.2)*	35.8 (35.4; 36.2)*		
CHA5118	Phl$^-$Prn$^-$Plt$^-$ Hcn$^-$Ofa$^-$	29.3 (28.9; 29.8)	32.6 (32.1; 33.1)		
CHA19	GacS$^-$			36.0 (35.3; 36.7)*	32.0 (31.5; 32.6)†
CHA89	GacA$^-$			32.6 (32.2; 33.1)†	33.8 (33.3; 34.3)
Control		NA	NA	NA	NA
		Experiment 1	Experiment 2		
CMR12a	Wild type	26.8 (26.5; 27.0)	29.2 (28.6; 29.8)		
Clp1	Ses$^-$	29.4 (29.1; 29.7)*	34.3 (33.9; 34.7)*		
ΔClp2	Ofa$^-$	26.4 (26.2; 26.7)	28.2 (27.8; 28.7)		
ΔClp2-clp1	Ofa$^-$ Ses$^-$	27.0 (26.7; 27.3)	30.0 (29.7; 30.4)		
ΔPhz	Phz$^-$	26.7 (26.5; 27.0)	29.8 (29.5; 30.2)		
ΔPhz-Clp1	Phz$^-$Ses$^-$	28.5 (28.2; 28.7)*	32.0 (31.7; 32.4)*		
ΔPhz-ΔClp2	Phz$^-$Ofa$^-$	26.1 (25.9; 26.3)†	29.1 (28.6; 29.6)		
ΔPhz-ΔClp2-Clp1	Phz$^-$Ofa$^-$Ses$^-$	28.8 (28.4; 29.2)*	31.9 (31.5; 32.3)*		
Control		NA	NA		
		Experiment 1	Experiment 2	Experiment 3	Experiment 4
PCL1391	Wild type	28.3 (28.0; 28.6)	29.3 (29.0; 29.7)	28.9 (28.4; 29.4)	26.5 (26.2; 26.8)
PCL1832	Clp1391$^-$	30.0 (29.6; 30.4)*	32.3 (31.8; 32.7)*	31.2 (30.7; 31.4)*	28.3 (27.8; 28.7)*
PCL1113	Phz$^-$	28.8 (28.4; 29.1)	30.0 (29.6; 30.5)		
PCL1123	GacS$^-$	29.2 (28.9; 29.5)*	30.5 (30.0; 31.0)*		
PCL5103	Hcn$^-$			32.4 (31.8; 33.0)*	27.4 (27.1; 27.8)*
Control		NA	NA	NA	NA

Lethal time (LT_{50}) values were calculated based on a generalized linear model (MASS package in R) from three replicates with 10 larvae per replicate. Confidence intervals (95%) for LT_{50} values are given in brackets. Marked values differed significantly from the LT_{50} value of the respective wild-type strain ($p < 0.05$). † significantly faster; * significantly slower; NA, no LT_{50} value was calculated, because mortality at the end of the experiment was <50%. Sterile 0.9% NaCl served as control (Control). Each strain was tested at least three times and results of at least two experiments are depicted. AprA, extracellular protease; Clp1391, cyclic lipopeptide of strain PCL1391; Fit, *P. fluorescens* insecticidal toxin; Gad, gluconate dehydrogenase; Gcd, glucose dehydrogenase; Hcn, hydrogen cyanide; Ofa, orfamide; Phl, 2,4-diacetylphloroglucinol; Phz, phenazine; Plt, pyoluteorin; Prn, pyrrolnitrin; Pvd, pyoverdine; Ses, sessilin.

In feeding experiments with *P. xylostella*, the Ofa$^-$ mutant of CHA0 was always killing significantly slower than the wild type (Figure 2D, Table 3) and larval mortality after 30 h was at 40.7% compared to 96.9% for the wild type (Figure 2D). Similar observations were made for the Ofa$^-$ mutant of CMR12a and the Clp$^-$ mutant of PCL1391, although the effects were not always significant for these strains (Figure 2E, F; Table 3). Similar to the results for injectable insecticidal activity, Ses seems to make a major contribution to oral insecticidal activity of strain CMR12a. In all repetitions of the experiment, larval death caused by a Ses$^-$ mutant was on average occurring at a later time point than death caused by the wild-type strain (Figure 2F, Table 3). Thus, after 28 h already 87.5% of larvae had died in the wild type treatment while mortality in the Ses$^-$ mutant treatment was only at 46.9% (Figure 2F).

As Ses, Ofa and Phzs are reported to influence each other's action (D'aes *et al.*, 2014; Olorunleke *et al.*, 2015), we also tested double and triple mutants of CMR12a. In *Galleria* injection experiments, a Ses$^-$Ofa$^-$ double mutant did not differ from the wild-type strain CMR12a, thus the Ses effect observed for the single mutant seems to disappear by the additional lack of Ofa. However, a triple mutant Phz$^-$Ofa$^-$Ses$^-$, which in addition to not producing the two Clps neither produces Phz, performed similar to the Ses$^-$ single mutant, killing *G. mellonella* larvae at a decreased pace compared to the wild type (Figure 2C, Table 3). In feeding experiments with larvae of *P. xylostella,* both the double mutant Ofa$^-$Ses$^-$ and the triple mutant Phz$^-$Ofa$^-$Ses$^-$ caused higher LT_{50} values than the wild type (Table 3). Furthermore, Ses and Ofa seem to have additive effects in oral infections as the Ofa$^-$Ses$^-$ double mutant and the Phz$^-$Ofa$^-$Ses$^-$ triple mutant showed not only significantly higher LT_{50} values compared to the Ofa$^-$ single mutant, but also a strong and consistent tendency towards higher LT_{50} values compared to the Ses$^-$ single mutant. Phz$^-$Ses$^-$ and Phz$^-$Ofa$^-$ double mutants did not kill more slowly than the respective Clp$^-$ single mutants (Table 3).

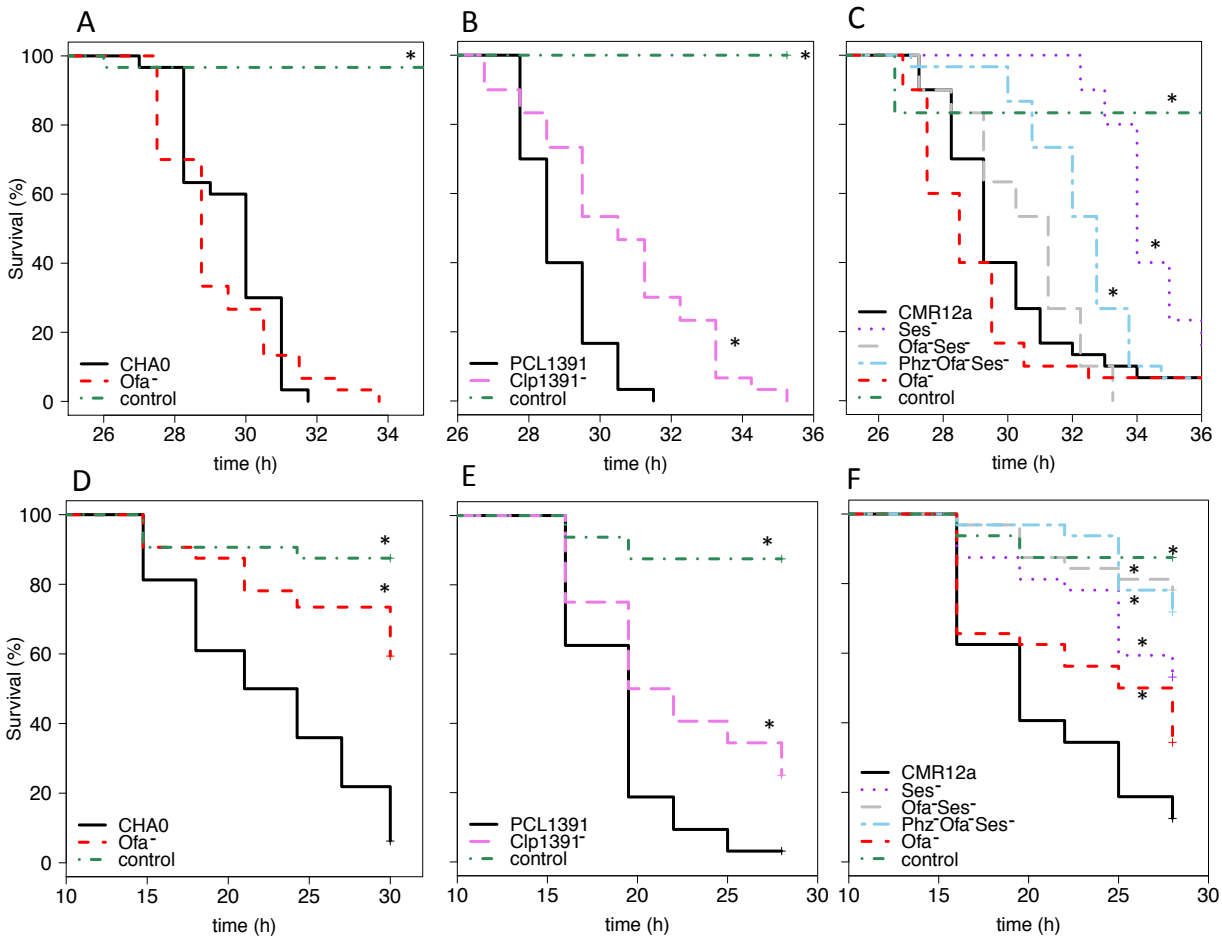


Figure 2 Cyclic lipopeptides contribute to insecticidal activity of three plant-beneficial pseudomonads. (A, B, C) Injectable activity against *Galleria mellonella*. 30 larvae per treatment were injected with 2 x 10^3 bacterial cells and survival was recorded hourly. **(D, E, F)** Oral activity against *Plutella xylostella*. For *Pseudomonas protegens* CHA0 **(D)** 64 larvae were exposed to artificial diet inoculated with 4 x 10^6 bacterial cells. In experiments with strains *Pseudomonas chlororaphis* PCL1391 **(E)** and *Pseudomonas* sp. CMR12a **(F)** 32 larvae were exposed to diet inoculated with 2 x 10^7 bacterial cells. Sterile 0.9% NaCl solution served as control. Treatments that differed significantly from their respective wild-type strain (Log-Rank test $p \leq 0.05$, Survival Package in R) are marked with an asterisk. LT_{50} values of the experiments presented above and of a repetition of the experiment are listed in Tables 2 and 3. Each strain was tested at least three times and similar results were obtained. Solid black line, wild-type strain; dashed red line, mutants deficient for orfamide production (Ofa⁻, CHA5101, CMR12a-ΔClp2), dashed pink line, mutant deficient for Clp1391 production (Clp1391⁻, PCL1832), dotted purple line, mutant deficient for sessilin production (Ses⁻, CMR12a-Clp1); dashed grey line, double mutant deficient for sessilin and orfamide production (Ses⁻Ofa⁻, CMR12a-ΔClp2-Clp1); dash-dot light blue line, triple mutant deficient for sessilin, orfamide and phenazine production (Ses⁻Ofa⁻Phz⁻, CMR12a-ΔPhz-ΔClp2-Clp1); dash-dot green line, 0.9% NaCl solution control.

Mutants defective in the production of Phl, Prn, Plt and Phz are not impaired in insecticidal activity

Pseudomonas sp. CMR12a and *P. chlororaphis* PCL1391 both produce phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN). Phzs are not only important for biocontrol of plant pathogenic fungi (Tambong and Höfte, 2001; Chin-A-Woeng *et al.*, 2003; D'aes *et al.*, 2011), but are further reported to be involved in biofilm formation and virulence (Mavrodi *et al.*, 2006; Price-Whelan *et al.*, 2006; Pierson and Pierson, 2010). However, in our experiments with Phz-deficient mutants of both strains, we did not find any reduction of virulence in infection assays with *G. mellonella* or *P. xylostella* larvae (Table 2, Table 3, Supplementary Figure S2).

P. protegens CHA0 has been intensively studied for its production of Phl, Plt and Prn, three metabolites contributing to antifungal activity of biocontrol pseudomonads on plant roots (Haas and Keel, 2003). We generated new in frame deletion mutants of CHA0 lacking biosynthesis genes needed for the production of these metabolites and screened them for insecticidal activity. All three mutants retained full toxicity in injection and feeding experiments (Table 2, Table 3, Supplementary Figure S2). Because the production of different antifungal metabolites in *P. protegens* is tightly interlinked and the loss of one antibiotic might be compensated by the overproduction of another antibiotic (Haas and Keel, 2003; Baehler *et al.*, 2005; Clifford *et al.*, 2015; Quecine *et al.*, 2015), we further created a triple mutant lacking the biosynthetic gene clusters for all three metabolites. In feeding experiments with *P. xylostella*, mortality caused by this mutant was not significantly different from that of the wild type (Table 3). However, a mutant that in addition to Phl, Prn and Plt also lacks Ofa and Hcn production exhibited strongly reduced virulence compared to the wild type (Table 3). It further displays reduced virulence compared to single mutants defective for either Ofa or Hcn indicating an additive effect of these two mutations in the five-fold mutant. In contrast, the toxicity of the five-fold mutant did not differ from that of the wild type in injection experiments (Table 2). The fact, that not even a reduction in virulence due to the lack of Hcn production was observed, cannot be explained at present, but might be attributable to possible adverse effects on the production of other, yet to be discovered factors.

Table 3 Lethal time 50 (LT_{50}) for *Plutella xylostella* larvae upon oral uptake of wild type and mutant *Pseudomonas* strains.

Strain	Phenotype	LT_{50} (h)		
		Experiment 1	Experiment 2	Experiment 3
CHA0	Wild type	24.8 (23.7; 25.8)	23 (21.8; 24.1)	20.8 (19.8; 21.8)
CHA89	GacA$^-$	NA		NA
CHA400	Pvd$^-$	26.8 (25.3; 28.2)		23.6 (22.7; 24.6)*
CHA1196	Gcd$^-$	21.9 (21.1; 22.7)†		23.6 (22.6; 24.6)*
CHA1197	Gad$^-$	26.0 (24.9; 27.2)		23.0 (22.1; 24.0)*
CHA805-g	AprA$^-$		22.1 (21.1; 23.2)	22.9 (21.8; 24.1)
CHA1241	Phl$^-$	24.3 (23.3; 25.4)		22.9 (21.9; 23.8)*
CHA5091	Prn$^-$	21.1 (20.0; 22.1)†		20.6 (19.6; 21.7)
CHA5092	Plt$^-$	27.1 (25.8; 28.4)		21.7 (20.8; 22.6)
CHA5098	Phl$^-$Prn$^-$Plt	26.4 (25.2; 27.6)		19.7 (18.7; 20.8)
CHA5101	Ofa$^-$	28.9 (27.7; 30.2)*		31.1 (29.3; 32.9)*
CHA5103	Hcn$^-$	27.8 (26.4; 29.3)*		23.7 (22.8; 24.6)*
CHA5118	Phl$^-$Prn$^-$Plt$^-$Hcn$^-$ Ofa$^-$	31.6 (30.4; 32.7)*		34.6 (32.6; 36.5)*
Control		NA		NA
		Experiment 1	Experiment 2	
CMR12a	Wild type	18.0 (15.6; 20.5)	21.0 (20.0; 22.0)	
Clp1	Ses$^-$	31.3 (28.0; 34.7)*	25.8 (23.8; 27.8)*	
ΔClp2	Ofa$^-$	23.5 (21.2; 25.9)*	20.9 (19.7; 22.1)	
ΔClp2-clp1	Ofa$^-$Ses$^-$	37.3 (32.9; 41.7)*	33.5 (29.2; 37.9)*	
ΔPhz	Phz$^-$	21.9 (20.4; 23.5)	20.8 (18.9; 22.7)	
ΔPhz-Clp1	Phz$^-$Ses$^-$	24.0 (22.3; 25.7)*	35.1 (31.5; 38.6)*	
ΔPhz-ΔClp2	Phz$^-$Ofa$^-$	20.1 (17.6; 22.6)	27.9 (26.3; 29.5)*	
ΔPhz-ΔClp2-Clp1	Phz$^-$Ofa$^-$Ses$^-$	37.2 (33.5; 40.9)*	30.7 (27.7; 33.7)*	
Control		NA	NA	
		Experiment 1	Experiment 2	Experiment 3
PCL1391	Wild type	17.1 (15.7; 18.5)	20.9 (19.2; 22.5)	27.9 (25.4; 30.5)
PCL1832	Clp1391$^-$	21.2 (19.4; 23.0)*	23.9 (21.5; 26.2)	
PCL1113	Phz$^-$	19.5 (18.1; 20.9)	19.9 (18.1; 21.7)	
PCL1123	GacS$^-$	37.6 (34.1; 41.2)*	34.0 (29.3; 38.8)*	
PCL5103	Hcn$^-$		22.9 (21.7; 24.2)	30.9 (28.4; 33.4)
Control		NA	NA	NA

Lethal time (LT_{50}) values were calculated based on a generalized linear model (MASS package in R) from eight replicates with 8 larvae per replicate in case of strain *P. protegens* CHA0 and from four replicates with eight larvae per replicate for strain *P. chlororaphis* PCL1391 and *Pseudomonas* sp. CMR12a. Confidence intervals (95%) for LT_{50} values are given in brackets. Marked values differed significantly from the LT_{50} value of the respective wild type strain ($p < 0.05$). † significantly faster; * significantly slower; NA, no LT_{50} value was calculated, because mortality at the end of the experiment was <50%. Sterile 0.9% NaCl served as control (Control). Each strain was tested at least three times and results of at least two experiments are depicted. AprA, extracellular protease; Clp1391, cyclic lipopeptide of strain PCL1391; Gad, gluconate dehydrogenase; Gcd, glucose dehydrogenase; Hcn, hydrogen cyanide; Ofa, orfamide; Phl, 2,4-diacetylphloroglucinol; Phz, phenazine; Plt, pyoluteorin; Prn, pyrrolnitrin; Pvd, pyoverdine; Ses, sessilin.

No significant contribution of Pvd, AprA, Gcd and Gad to insect virulence

Finally, mutants of strain CHA0 deficient for the iron chelator Pvd, the two enzymes Gcd and Gad, which are involved in glucose utilization, or the AprA protease were tested in the systemic and oral insect infection assays, but none of them was found to behave differently from the wild type (Table 2, Table 3). Although some of the strains were found to kill significantly faster or slower in one experiment, this result was not observed in other repetitions of the experiment. In some cases, even contradictory results were obtained in different repetitions, as for instance for the behavior of the Gcd$^-$ mutant in *P. xylostella* experiments (Table 3).

Gac-Regulation

The GacA/GacS regulatory system is controlling a plethora of processes in fluorescent pseudomonads. *gac* mutants are deficient for the production of antifungal metabolites and generally strongly reduced in biocontrol against root pathogens (Lapouge *et al.*, 2008; Hassan *et al.*, 2010). Furthermore, it was shown that a *gacA* mutant is also reduced in oral insecticidal activity (Olcott *et al.*, 2010; Ruffner *et al.*, 2013; Flury *et al.*, 2016; Loper *et al.*, 2016). Here, we tested Gac$^-$ mutants of CHA0 and PCL1391 in feeding assays and, for the first time, also in injection experiments. In oral infections of *P. xylostella*, a defect in the GacA/GacS regulatory system for both strains resulted in a drastic reduction of larval mortality and killing speed (Table 3, Supplementary Figure S2). This might be partly caused by the failure of these mutants to produce the above identified virulence factors, Hcn and Ofa/Clp1391 known to be under Gac control (Laville *et al.*, 1992; Hassan *et al.*, 2010). In contrast to the results obtained in feeding experiments, the effect of a *gacS* mutation in PCL1391 was very small in injection assays with *G. mellonella* larvae, i.e. resulting in a delay of killing of only about one hour compared to the wild type (Table 2). Moreover, for GacA$^-$ and GacS$^-$ mutants of CHA0 not even a consistent significant effect was observed (Table 2). In summary, the GacA/GacS regulatory system seems to be crucial for oral insecticidal activity of *P. chlororaphis* subgroup bacteria, but of limited importance when bacteria are directly injected into the hemocoel.

Biosynthesis genes for antimicrobial compounds are expressed in insects

As a lack of neither Phl nor Prn or Plt affected virulence of *P. protegens* CHA0, we wondered whether these metabolites are actually produced in insects. We, therefore, checked the expression of the biosynthetic genes for these metabolites during infections upon injection of *G. mellonella* and during oral infections of *P. xylostella* by a qualitative PCR approach. Expression of *phlD*, *prnD* and *pltA* was detected at early (20 h) and late time points (30 h) during systemic infections of *G. mellonella* larvae (Table 4, Supplementary Figure S3). *prnD* and *pltA* were further expressed at both time points during oral infections, although not in all repetitions of the experiments (Table 4, Supplementary Figure S3). On the other hand, expression of *phlD* was only observed in dead and never in infected, but still living *P. xylostella* larvae.

We also tested the expression of genes required for the production of the insecticidal factors Ofa, Hcn and FitD. In infections upon injection of *G. mellonella fitD* and *hcnA* were consistently expressed at both time points, while expression of *ofaA* was only detected after 30 hours (Table 4). In oral infections all three genes were expressed in at least two out of tree experiments at the late time point. At time point 20 h, however, *ofaA* was never expressed and *fitD* and *hcnA* expression could only be detected in one out of three experiments (Table 4, Supplementary Figure S3).

Table 4 Expression of biosynthetic genes for antifungal metabolites during insect infection by *Pseudomonas protegens* CHA0

	16s		*ofaA*		*phlD*		*fitD*		*prnD*		*hcnA*		*pltA*	
	i	o	i	o	i	o	i	o	i	o	i	o	i	o
20 h	3	3	0	0	3	0	3	1	2	2	3	1	3	1
30 h	3	3	3	2	2	0	3	3	2	3	3	2	3	1
Dead	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Galleria mellonella larvae were infected systemically by injection (i) of 2 x 10^3 cells of *P. protegens* CHA0 into the hemocoel. *Plutella xylostella* larvae were infected orally (o) by feeding artificial diet inoculated with 4 x 10^6 cells of *P. protegens* CHA0. For *G. mellonella* infections, hemocoel was collected 20 h and 30 h post injection (living larvae) and after 42 h when larvae had succumbed to infection (Dead). For *P. xylostella* infections, entire larvae were collected after 20 h and 30 h and when death occurred (Dead). Total RNA was extracted from insect tissue, converted into cDNA and gene expression for the indicated genes was checked by PCR. Because results for some of the genes varied, a summary of the outcome of three experiments is presented and numbers indicate how many times (out of three) expression of the respective gene was detected. A *Pseudomonas* specific primer for the *16s rRNA* gene was used to detect presence of the bacteria. In non-infected control larvae, neither expression of the *Pseudomonas 16s rRNA* gene nor of any of the other genes was detected. Gel pictures for one injection and one feeding experiment are presented in Supplementary Figure S3.

DISCUSSION

This study presents an extensive mutational analysis with the aim to identify antimicrobial compounds that contribute to insecticidal activity of three different *Pseudomonas* biocontrol strains, *P. protegens* CHA0, *P. chlororaphis* PCL1391 and *Pseudomonas* sp. CMR12a. We identified Hcn as an important toxicity factor in strains CHA0 and PCL1391. In addition, mutants impaired in production of various Clps showed reduced insect toxicity in all three strains. Furthermore, we provide the first investigation on antibiotic production by a fluorescent pseudomonad in insect hosts. In summary, our results indicate that, indeed, certain antifungal compounds also contribute to anti-insect activity. To illustrate the knowledge on antimicrobial and insecticidal compounds produced by *P. chlororaphis* subgroup bacteria generated in this study and reported previously, we present an overview of major exoproducts and whether they are involved in activity against microbes or insects or both (Figure 3).

P. protegens CHA0 and *P. chlororaphis* PCL1391 lacking Hcn production showed a significant decrease of virulence when injected into the hemocoel of *G. mellonella* larvae. However, the effect was smaller and for PCL1391 there was only a tendency towards reduced toxicity when bacteria were ingested by *P. xylostella*. Cyanide is known to be toxic to most organisms as it inhibits the cytochrome-c oxidase, which is part of the mitochondrial respiratory chain (Way, 1984). Accordingly, cytochrome-c oxidase activity of termites was inhibited when they were exposed to Hcn produced by CHA0, finally causing insect death (Devi and Kothamasi, 2009). In injection experiments, cyanide was found to contribute to virulence of *Pseudomonas aeruginosa* PAO1 towards *D. melanogaster* (Broderick *et al.*, 2008). In contrast, in a recent study by Loper and colleagues (2016) a lack of Hcn production in *P. protegens* Pf-5 did not impair its oral insecticidal activity against *D. melanogaster*. Both results are in line with our observation that a lack of Hcn productionreduces toxicity of CHA0 and PCL1391 mainly in the hemolymph. We, thus, hypothesize that Hcn comes into play at a late stage of infection, once the bacteria already entered the hemolymph. Accordingly, at early stage of oral infections, when the bacteria assumingly still are in the gut, the Hcn biosynthesis gene *hcnA* of CHA0 was only expressed in one out of three experiments. However, strong expression of *hcnA* was observed in all experiments and at all stages when bacteria were injected into the larvae.

We tested mutants deficient for different Clps, i.e. orfamide A, orfamide B, Clp1391 and sessilin, and our results indicate that all of them contribute to insect toxicity exhibited by their producer strains. The strongest effect was found for sessilin, which is to our best knowledge the first report on the role of a tolaasin-like Clp in insect infection. The results obtained for *P. protegens* CHA0 are in line with former reports that orfamide A of strains F6 and Pf-5 contributes to toxicity against aphids and *D. melanogaster*, respectively (Jang *et al.*, 2013; Loper *et al.*, 2016). The main orfamide produced by CMR12a is orfamide B (Ma *et al.*, 2016), and we provide first evidence that also this type of orfamide is contributing to insecticidal activity. The large size of the genes encoding the non-ribosomal peptide synthases responsible for the biosynthesis of Clps makes complementation of mutants extremely difficult. However, we believe that the detection of reduced toxicity by Clp$^-$ mutants in three different strains, strongly suggests an important role for these molecules during insect infections. Still, how Clps contribute to insect infections, their target and mode of action remains speculative.

Clps are reported to be involved in many biological functions, such as motility, biofilm formation, protection against predators and antagonism (Raaijmakers *et al.*, 2010). On plants Clps produced by plant-beneficial bacteria were found to induce resistance and to contribute to plant protection against root pathogenic fungi (Raaijmakers *et al.*, 2006; D'aes *et al.*, 2010; D'aes *et al.*, 2011; Olorunleke *et al.*, 2015). For instance the Clps massetolide A and viscosin of *P. fluorescens* SS101 and *P. fluorescens* SBW25, respectively, are able to cause lysis of zoospores of the oomycete pathogen *Phytophthora infestans*, the causal agent of tomato and potato late blight (de Souza *et al.*, 2003; de Bruijn *et al.*, 2007). In plant pathogenic *Pseudomonas*, Clps contribute to virulence by inserting into the host plasma membrane, forming pores, which leads to cell death and thus necrotic symptoms (D'aes *et al.*, 2010). During infection, insecticidal pseudomonads do not remain in the gut, but cause fatal septicemia (Kupferschmied *et al.*, 2013). Hence, upon oral uptake, bacteria have not only to survive the adverse conditions of the gut milieu and the insect immune response, but also to overcome the peritrophic membrane and the gut epithelial cells to gain access to the hemocoel (Vallet-Gely *et al.*, 2008). Clps might be important in different ways during the infection of insects, on one hand for motility and biofilm formation during insect colonization and on the other hand to effectively damage the peritrophic membrane and/ or insect blood cells, such as phagocytic hemocytes,

involved in immunity against intruding pathogens (Lemaitre and Hoffmann, 2007). Recently, Sood and colleagues (2014) reported a role for Clps in insect infections. They identified paenilarvins, iturin-like Clps, in *Paenibacillus larvae*, a deadly bee pathogen causing American foulbrood. Paenilarvins, which also have activity against various filamentous fungi and yeasts, were found to cause significant mortality when fed to bee larvae (Sood *et al.*, 2014; Müller *et al.*, 2015). However, as for the Clps studied here, it is still elusive by which mechanisms paenilarvins affect insect larvae.

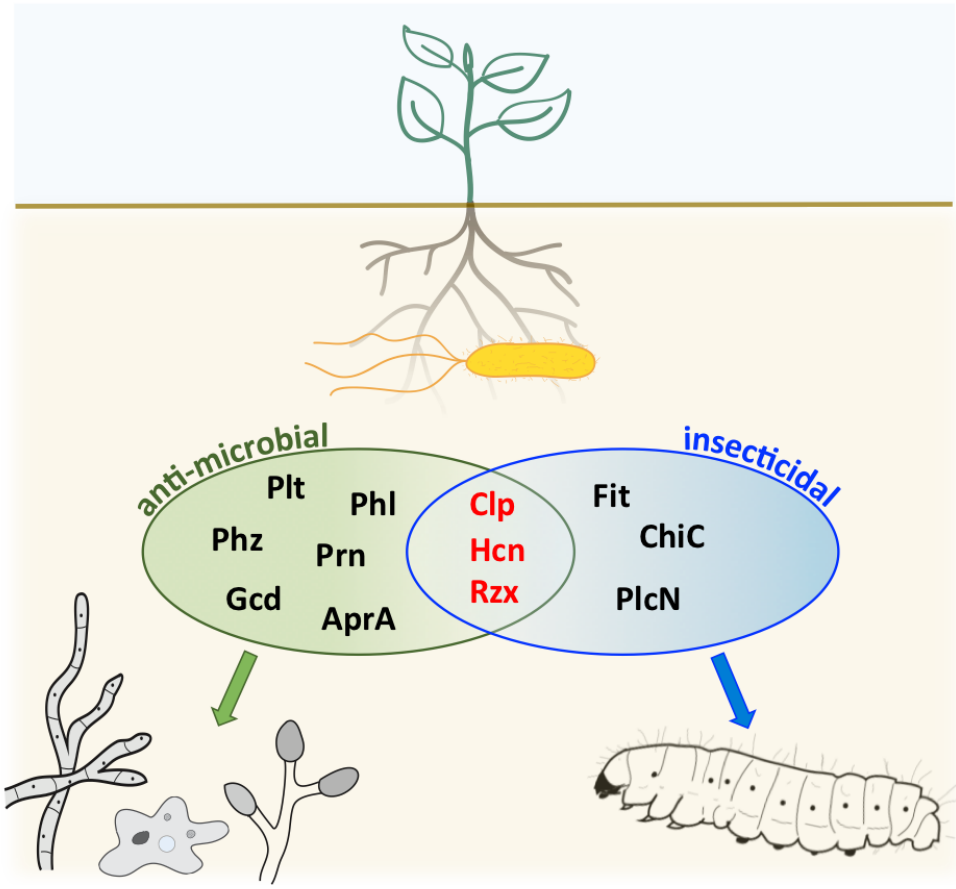


Figure 3 Overview of exoproducts contributing to the antimicrobial and insecticidal activity of bacteria of the *Pseudomonas chlororaphis* subgroup. Compounds involved in activity against both microorganisms as well as insects are depicted in red. AprA; protease AprA; ChiC, chitinase C; Clp, cyclic lipopeptide; Fit, *P. fluorescens* insecticidal toxin; Gcd, Glucose dehydrogenase; Hcn, hydrogen cyanide; Phl, 2,4-diacetylphloroglucinol; Phz, phenazine; PlcN, phospholipase C; Plt, pyoluteorin; Prn, pyrrolnitrin; Rzx, rhizoxin.

CMR12a is the only herein tested strain that produces two types of Clps, which is atypical for plant-beneficial pseudomonads. For plant-pathogenic *Pseudomonas* strains, however, which mostly produce one Clp each of the tolaasin and the syringomycin group (D'aes *et al.*, 2010) it was suggested that the two Clps act synergistically, one as a toxin and the other as surfactant (Iacobellis *et al.*, 1992; Batoko *et al.*, 1998; Bender *et al.*, 1999). Since our data imply that both Clps of CMR12a are involved in virulence towards insects, one could hypothesize a similar interplay of Ses and Ofa as described for Clps of *Pseudomonas syringae*. Motility and biofilm formation are often connected to bacterial pathogenicity. The Ofa$^-$ mutants of CHA0 and CMR12a are impaired in swarming motility while the Ses$^-$ mutant shows reduced biofilm formation (D'aes *et al.*, 2014). In the Ofa$^-$Ses$^-$ double mutant of CMR12a the two mutations seem to partially compensate each other since the double mutant e.g. does produce more biofilm than the Ses$^-$ single mutant (D'aes *et al.*, 2014). This might explain our observation that the Ses$^-$ Ofa$^-$ mutant is killing *G. mellonella* larvae more rapidly than the Ses$^-$ single mutant. In general, a tight regulation between mobility and settlement seems to be crucial for full virulence of the herein studied bacteria against insects. While orfamides seem to be mainly important in oral infections of *P. xylostella*, a lack of Clp1391 and Ses also hampered infections of *G. mellonella* larvae upon injection. This may be due to different functions of structurally different Clps during the gut or the hemolymph phases of infection. However, it could be also attributed to a possible host specificity of certain toxicity factors, which cannot be excluded as we used two different model insects for injection and feeding experiments.

Besides Hcn and Clp, none of the other antimicrobial compounds investigated affected virulence in our test systems. The impact of phenazines on insecticidal activity of fluorescent pseudomonads was studied for the first time and the fact that Phz-deficient mutants of both CMR12a and PCL1391 did not differ in their toxicity from the respective wild type indicates that Phzs do not play a crucial role in insect infections by strains of the *P. chlororaphis* subgroup. Likewise, a pyocyanin deficient mutant of *P. aeruginosa* PAO1 was not reduced in virulence when injected into the hemocoel of the silkworm *Bombyx mori* (Chieda *et al.*, 2008). Since Phzs are reported to contribute to *P. aeruginosa* virulence, e.g. during lung infections (Pierson and Pierson, 2010), Chieda and colleagues hypothesized that a possible pyocyanin effect might have been masked in their experiments as the investigated pyocyanin mutant was still able to produce

other Phzs, such as PCA and PCN. However, the Phz$^-$ mutants of PCL1391 and CMR12a are defective for the production of all Phzs naturally produced by their respective wild-type strains and still no reduction of virulence compared to the wild type could be observed.

Furthermore, Phl, Prn and Plt show broad toxicity towards different organisms (Gross and Loper, 2009), but CHA0 mutants impaired in their production killed insects at the same pace as the wild type. This is in line with recent results by Loper and colleagues (2016) who neither found an effect of these antimicrobial compounds in oral toxicity of *P. protegens* Pf-5 towards *D. melanogaster.* However, we further studied the expression of antibiotic biosynthesis genes and our results imply that during infections by *P. protegens* CHA0 not only the biosynthetic genes for the insect toxicity factors Hcn and Ofa are expressed, but also the genes required for Phl, Prn and Plt production. This indicates that these factors, although being dispensable for full virulence towards insects, still may have a function during insect colonization. As *prn* and *plt* biosynthetic genes are already expressed at early time-points in oral infections, when bacteria presumably are still colonizing the gut, it could be hypothesized that the respective compounds give an advantage in competition with other microbes present in the digestive tract of insects. This effect might thus be much more relevant in infections of natural insect populations harboring a more diverse microflora than the herein tested laboratory-grown animals. After larvae have succumbed to infection by *P. protegens* CHA0, the bacteria can use the cadaver as an optimal substrate for growth. At this stage, biosynthesis genes for all known antimicrobial compounds are expressed, which could again give a competitive advantage over other microbes trying to intrude the nutrient-rich insect substrate. Similarly, the entomopathogens *Photorhabdus* and *Xenorhabdus* produce numerous secondary metabolites to inhibit the growth of other microbes in the insect cadaver (Nielsen-LeRoux *et al.*, 2012).

The exoprotease AprA adds to the biocontrol potential of CHA0 against the root knot nematode *Meloidogyne incognita* (Siddiqui *et al.*, 2005). Further, AprA of the insect pathogen *Pseudomonas entomophila* is reported to contribute to virulence against *D. melanogaster* and was suggested to protect the bacteria against the insect immune response (Liehl *et al.*, 2006). In the insect injection and feeding assays presented here, an *aprA* mutant of CHA0 was as virulent as the wild type. Similar results were obtained recently with an *aprA* mutant of Pf-5 (Loper *et al.*, 2016) and an *aprX* mutant of CHA0 in

oral infection assays (Flury *et al.*, 2016). All these findings suggest that these exoproteases do not play an important role in insect infections by *P. protegens*. Similarly, a pyoverdine mutant was found to be as virulent as the wild type CHA0 confirming the results recently obtained with a *pvd* mutant of Pf-5 (Loper *et al.*, 2016).

As demonstrated earlier (Olcott *et al.*, 2010; Ruffner *et al.*, 2013; Flury *et al.*, 2016; Loper *et al.*, 2016) Gac$^-$ mutants were found to exhibit drastically reduced toxicity in oral insect infections. However, this was not the case in the injection experiments carried out in the present study. Hence, although factors positively controlled by the GacS/GacA regulatory system seem to be crucial for insect infection through the intestine, they seem to be irrelevant or compensated by other factors once the bacterium has entered the hemolymph.

In summary, the presented research enriches our knowledge on factors that are important for insecticidal activity of plant-beneficial pseudomonads. We were searching for compounds contributing to dual activity against microbes and insects and discovered that in fact Hcn and Clps exhibit such versatile functions (Figure 3). Our study has unraveled the cyclic lipopeptides orfamides A and B, Clp1391 and sessilin as further strain-specific insect virulence factors besides rhizoxin in plant-beneficial pseudomonads. Hcn can be added to the anti-insect arsenal common to all strains of the *P. chlororaphis* subgroup, i.e. Fit toxin, chitinase C, and phospholipase C (Flury *et al.*, 2016). Nevertheless, insecticidal activity seems to be orchestrated by various factors and many of them are presumably still awaiting their discovery until a concluding picture on insect virulence in the *P. chlororaphis* subgroup can be drawn (Keel, 2016). Moreover, how the herein described toxicity factors intervene during insect infection, their targets and modes of action warrant further investigation. Besides the identification of new toxicity factors our study is the first to investigate expression of biosynthesis genes of antibiotic compounds during insect infection and our results indicate that antifungal metabolites may not only be of importance on plant roots, but presumably also play some role in insect hosts. This points to an even larger versatility of these compounds than assumed so far. In general, *P. protegens* CHA0, *P. chlororaphis* PCL1391 and *Pseudomonas* sp. CMR12a seem to possess an arsenal of weapons to compete in different habitats ranging from roots to insects and maybe even other yet to be discovered niches. It will be a fascinating task to further explore, how they switch

between their different life-styles as plant-colonizing beneficial bacteria and insect-colonizing pathogenic bacteria.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHORS AND CONTRIBUTORS

PF, SW, GB, CK and MM designed the research; GB, MH, CK and MM supervised the study; PF, MP, PK, KK, ZM (Ghent University) and SW generated and characterized the mutants; PF, NA, NH, SS, PV and ZM (ETH Zürich) performed the insect experiments; PV and FD performed the gene expression analysis; PF analysed the data and wrote the paper together with SW, GB, MH, CK and MM. All authors critically revised the manuscript and approved the final version.

FUNDING

The study was funded by grants of the Swiss National Foundation for Scientific Research SNSF (Projects 31003A-138248, 31003A-159520, NRP68 406840_143141 and 406840_161904. KK was supported by a Scholarships for Foreign Students of the Swiss Government. PF was supported with a 'Mobility Grant for Research Stays' granted by the Swiss Plant Science Web. Z.M. (Ghent University) was supported by scholarships from China Scholarship Council (CSC, No. 201204910376) and a special research fund (Bijzonder Onderzoeksfonds, BOF) from Ghent University.

ACKNOWLEDGMENTS

We thank Ellen Lagendijk for isolation and identification of the transposon mutants of *P. chlororaphis* PCL1391. Furthermore, we acknowledge Syngenta Crop Protection (Stein, Switzerland) for generously providing *P. xylostella* larvae.

REFERENCES

Altschul S. F., Madden T. L., Schaffer A. A., Zhang J., Zhang Z., Miller W. *et al.* (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25**:** 3389-3402.

Baehler E., Bottiglieri M., Pechy-Tarr M., Maurhofer M., Keel C. (2005). Use of green fluorescent protein-based reporters to monitor balanced production of antifungal compounds in the biocontrol agent *Pseudomonas fluorescens* CHA0. *J Appl Microbiol* 99**:** 24-38.

Bassarello C., Lazzaroni S., Bifulco G., Lo Cantore P., Iacobellis N. S., Riccio R. *et al.* (2004). Tolaasins A–E, five new lipodepsipeptides produced by *Pseudomonas tolaasii*. *J Nat Prod* 67**:** 811-816.

Batoko H., de Kerchove d'Exaerde A., Kinet J. M., Bouharmont J., Gage R. A., Maraite H. *et al.* (1998). Modulation of plant plasma membrane H+-ATPase by phytotoxic lipodepsipeptides produced by the plant pathogen *Pseudomonas fuscovaginae*. *Biochim Biophys Acta* 1372**:** 216-226.

Bender C. L., Alarcon-Chaidez F., Gross D. C. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol Mol Biol Rev* 63**:** 266-292.

Bergmark L., Poulsen P. H., Al-Soud W. A., Norman A., Hansen L. H., Sorensen S. J. (2012). Assessment of the specificity of *Burkholderia* and *Pseudomonas* qPCR assays for detection of these genera in soil using 454 pyrosequencing. *FEMS Microbiol Lett* 333**:** 77-84.

Bertani G. (1951). Studies on lysogenesis.1. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 62**:** 293-300.

Broderick K. E., Chan A., Balasubramanian M., Feala J., Reed S. L., Panda M. *et al.* (2008). Cyanide produced by human isolates of *Pseudomonas aeruginosa* contributes to lethality in *Drosophila melanogaster*. *J Infect Dis* 197**:** 457-464.

Castric K. F., Castric P. A. (1983). Method for rapid detection of cyanogenic bacteria. *Appl Environ Microbiol* 45**:** 701-702.

Chieda Y., Iiyama K., Lee J. M., Kusakabe T., Yasunaga-Aoki C., Shimizu S. (2008). Inactivation of pyocyanin synthesis genes has no effect on the virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm, *Bombyx mori*. *FEMS Microbiol Lett* 278**:** 101-107.

Chin-A-Woeng T. F. C., Bloemberg G. V., van der Bij A. J., van der Drift K., Schripsema J., Kroon B. *et al.* (1998). Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant-Microbe Interact* 11**:** 1069-1077.

Chin-A-Woeng T. F. C., Bloemberg G. V., Lugtenberg B. J. J. (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol* 157**:** 503-523.

Clifford J. C., Buchanan A., Vining O., Kidarsa T. A., Chang J. H., McPhail K. L. *et al.* (2015). Phloroglucinol functions as an intracellular and intercellular chemical messenger influencing gene expression in *Pseudomonas protegens*. *Environ Microbiol* 10.1111/1462-2920.13043.

D'aes J., De Maeyer K., Pauwelyn E., Hofte M. (2010). Biosurfactants in plant-*Pseudomonas* interactions and their importance to biocontrol. *Environmental microbiology reports* 2**:** 359-372.

D'aes J., Hua G. K., De Maeyer K., Pannecoucque J., Forrez I., Ongena M. *et al.* (2011). Biological control of *Rhizoctonia* root rot on bean by phenazine- and cyclic lipopeptide-producing *Pseudomonas* CMR12a. *Phytopathology* 101**:** 996-1004.

D'aes J., Kieu N. P., Leclere V., Tokarski C., Olorunleke F. E., De Maeyer K. *et al.* (2014). To settle or to move? The interplay between two classes of cyclic lipopeptides in the biocontrol strain *Pseudomonas* CMR12a. *Environ Microbiol* 16**:** 2282-2300.

de Bruijn I., de Kock M. J., Yang M., de Waard P., van Beek T. A., Raaijmakers J. M. (2007). Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species. *Mol Microbiol* 63**:** 417-428.

de Souza J. T., de Boer M., de Waard P., van Beek T. A., Raaijmakers J. M. (2003). Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. *Appl Environ Microbiol* 69**:** 7161-7172.

de Werra P., Pechy-Tarr M., Keel C., Maurhofer M. (2009). Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Appl Environ Microbiol* 75**:** 4162-4174.

Devi K. K., Kothamasi D. (2009). *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting cytochromecoxidase of the termite respiratory chain. *FEMS Microbiol Lett* 300**:** 195-200.

Ditta G., Stanfield S., Corbin D., Helinski D. R. (1980). Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proceedings of the National Academy of Sciences of the United States of America* 77**:** 7347-7351.

Flury P., Aellen N., Ruffner B., Pechy-Tarr M., Fataar S., Metla Z. *et al.* (2016). Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics. *The ISME journal* 10**:** 2527-2542.

Gomila M., Peña A., Mulet M., Lalucat J., García-Valdés E. (2015). Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 6**:** 214.

Gross H., Stockwell V. O., Henkels M. D., Nowak-Thompson B., Loper J. E., Gerwick W. H. (2007). The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. *Chem Biol* 14**:** 53-63.

Gross H., Loper J. E. (2009). Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat Prod Rep* 26**:** 1408-1446.

Haas D., Keel C. (2003). Regulation of antibiotic production in root-colonizing *Peudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol* 41**:** 117-153.

Hassan K. A., Johnson A., Shaffer B. T., Ren Q., Kidarsa T. A., Elbourne L. D. *et al.* (2010). Inactivation of the GacA response regulator in *Pseudomonas fluorescens* Pf-5 has far-reaching transcriptomic consequences. *Environ Microbiol* 12**:** 899-915.

Iacobellis N. S., Lavermicocca P., Grgurina I., Simmaco M., Ballio A. (1992). Phytotoxic properties of *Pseudomonas syringae* pv. syringae toxins. *Physiol Mol Plant Pathol* 40**:** 107-116.

Jain D. K., Collinsthompson D. L., Lee H., Trevors J. T. (1991). A drop-collapsing test for screening surfactant-producing microorganisms. *J Microbiol Methods* 13**:** 271-279.

Jang J. Y., Yang S. Y., Kim Y. C., Lee C. W., Park M. S., Kim J. C. *et al.* (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J Agric Food Chem* 61**:** 6786-6791.

Jousset A., Schuldes J., Keel C., Maurhofer M., Daniel R., Scheu S. *et al.* (2014). Full-genome sequence of the plant growth-promoting bacterium *Pseudomonas protegens* CHA0. *Genome Announc* 2**:** e00322-00314.

Keel C., Voisard C., Berling C. H., Kahr G., Défago G. (1989). Iron sufficiency, a prerequisite for the suppression of tobacco black root-rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* 79**:** 584-589.

Keel C., Schnider U., Maurhofer M., Voisard C., Laville J., Burger U. *et al.* (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0 - Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant-Microbe Interact* 5**:** 4-13.

Keel C. (2016). A look into the toolbox of multi-talents: insect pathogenicity determinants of plant-beneficial pseudomonads. *Environ Microbiol* 18**:** 3207-3209.

Kidarsa T. A., Shaffer B. T., Goebel N. C., Roberts D. P., Buyer J. S., Johnson A. *et al.* (2013). Genes expressed by the biological control bacterium *Pseudomonas protegens* Pf-5 on seed surfaces under the control of the global regulators GacA and RpoS. *Environ Microbiol* 15**:** 716-735.

King E. O., Ward M. K., Raney D. E. (1954). 2 simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* 44**:** 301-307.

Kupferschmied P., Maurhofer M., Keel C. (2013). Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Frontiers in plant science* 4**:** 287.

Kupferschmied P., Péchy-Tarr M., Imperiali N., Maurhofer M., Keel C. (2014). Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. *PLoS Path* 10**:** e1003964.

Lapouge K., Schubert M., Allain F. H., Haas D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol Microbiol* 67**:** 241-253.

Laville J., Voisard C., Keel C., Maurhofer M., Defago G., Haas D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings of the National Academy of Sciences of the United States of America* 89**:** 1562-1566.

Lemaitre B., Hoffmann J. (2007). The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 25**:** 697-743.

Liehl P., Blight M., Vodovar N., Boccard F., Lemaitre B. (2006). Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. *PLoS Path* 2**:** e56.

Loper J. E., Hassan K. A., Mavrodi D. V., Davis E. W., 2nd, Lim C. K., Shaffer B. T. *et al.* (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet* 8**:** e1002784.

Loper J. E., Henkels M. D., Rangel L. I., Olcott M. H., Walker F. L., Bond K. L. *et al.* (2016). Rhizoxin, orfamide A, and chitinase production contribute to the toxicity of *Pseudomonas protegens* strain Pf-5 to *Drosophila melanogaster*. *Environ Microbiol* 18**:** 3509-3521.

Ma Z., Geudens N., Kieu N. P., Sinnaeve D., Ongena M., Martins J. C. *et al.* (2016). Biosynthesis, chemical structure, and structure-activity relationship of orfamide lipopeptides produced by *Pseudomonas protegens* and related species. *Front Microbiol* 7**:** 382.

Martinez-Garcia E., de Lorenzo V. (2011). Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Environ Microbiol* 13**:** 2702-2716.

Maurhofer M., Keel C., Schnider U., Voisard C., Haas D., Defago G. (1992). Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology* 82**:** 190-195.

Maurhofer M., Keel C., Haas D., Defago G. (1995). Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHA0 with enhanced antibiotic production. *Plant Pathol* 44**:** 40-50.

Mavrodi D. V., Blankenfeldt W., Thomashow L. S. (2006). Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annu Rev Phytopathol* 44**:** 417-445.

Müller S., Garcia-Gonzalez E., Genersch E., Süssmuth R. D. (2015). Involvement of secondary metabolites in the pathogenesis of the American foulbrood of honey bees caused by *Paenibacillus larvae*. *Nat Prod Rep* 32**:** 765-778.

Neidig N., Paul R. J., Scheu S., Jousset A. (2011). Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. *Microb Ecol* 61**:** 853-859.

Nielsen-LeRoux C., Gaudriault S., Ramarao N., Lereclus D., Givaudan A. (2012). How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus*/*Photorhabdus* occupy their hosts. *Curr Opin Microbiol* 15**:** 220-231.

Nisr R. B., Russell M. A., Chrachri A., Moody A. J., Gilpin M. L. (2011). Effects of the microbial secondary metabolites pyrrolnitrin, phenazine and patulin on INS-1 rat pancreatic beta-cells. *FEMS Immunol Med Microbiol* 63**:** 217-227.

Olcott M. H., Henkels M. D., Rosen K. L., Walker F. L., Sneh B., Loper J. E. *et al.* (2010). Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. *PloS one* 5**:** e12504.

Olorunleke F. E., Hua G. K., Kieu N. P., Ma Z., Hofte M. (2015). Interplay between orfamides, sessilins and phenazines in the control of *Rhizoctonia diseases* by *Pseudomonas* sp. CMR12a. *Environmental microbiology reports* 7**:** 774-781.

Péchy-Tarr M., Bruck D. J., Maurhofer M., Fischer E., Vogne C., Henkels M. D. *et al.* (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ Microbiol* 10**:** 2368-2386.

Péchy-Tarr M., Borel N., Kupferschmied P., Turner V., Binggeli O., Radovanovic D. *et al.* (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ Microbiol* 15**:** 736-750.

Perneel M., Heyrman J., Adiobo A., De Maeyer K., Raaijmakers J. M., De Vos P. *et al.* (2007). Characterization of CMR5c and CMR12a, novel fluorescent *Pseudomonas* strains from the cocoyam rhizosphere with biocontrol activity. *J Appl Microbiol* 103**:** 1007-1020.

Pierson L. S., 3rd, Pierson E. A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl Microbiol Biotechnol* 86**:** 1659-1670.

Price-Whelan A., Dietrich L. E., Newman D. K. (2006). Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* 2**:** 71-78.

Quecine M. C., Kidarsa T. A., Goebel N. C., Shaffer B. T., Henkels M. D., Zabriskie T. M. *et al.* (2015). An interspecies signaling system mediated by fusaric acid has parallel effects on antifungal metabolite production by *Pseudomonas protegens* strain Pf-5 and antibiosis of *Fusarium* spp. *Appl Environ Microbiol* 82**:** 1372-1382.

Raaijmakers J. M., de Bruijn I., de Kock M. J. (2006). Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Molecular plant-microbe interactions : MPMI* 19**:** 699-710.

Raaijmakers J. M., De Bruijn I., Nybroe O., Ongena M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol Rev* 34**:** 1037-1062.

Ruffner B., Péchy-Tarr M., Ryffel F., Hoegger P., Obrist C., Rindlisbacher A. *et al.* (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ Microbiol* 15**:** 751-763.

Ruffner B., Pechy-Tarr M., Hofte M., Bloemberg G., Grunder J., Keel C. *et al.* (2015). Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* 16**:** 609.

Sambrook J., Russel D. W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: New York.

Siddiqui I. A., Haas D., Heeb S. (2005). Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. *Appl Environ Microbiol* 71**:** 5646-5649.

Simon R., Priefer U., Puhler A. (1983). A broad host range mobilization system for invivo genetic-engineering - transposon mutagenesis in Gram-negative bacteria. *Bio-Technol* 1**:** 784-791.

Sood S., Steinmetz H., Beims H., Mohr K. I., Stadler M., Djukic M. *et al.* (2014). Paenilarvins: Iturin family lipopeptides from the honey bee pathogen *Paenibacillus larvae*. *ChemBioChem* 15**:** 1947-1955.

Stutz E. W., Défago G., Kern H. (1986). Naturally-occuring fluorescent pseudomonads involved in suppression of black root-rot of tobacco. *Phytopathology* 76**:** 181-185.

Tambong J. T., Höfte M. (2001). Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. *Eur J Plant Pathol* 107**:** 511-521.

Vallet-Gely I., Lemaitre B., Boccard F. (2008). Bacterial strategies to overcome insect defences. *Nat Rev Microbiol* 6**:** 302-313.

Voisard C., Keel C., Haas D., Defago G. (1989). Cyanide production by *Pseudomonas fluorescens* helps suppress black root-rot of tobacco under gnotobiotic conditions. *EMBO J* 8**:** 351-358.

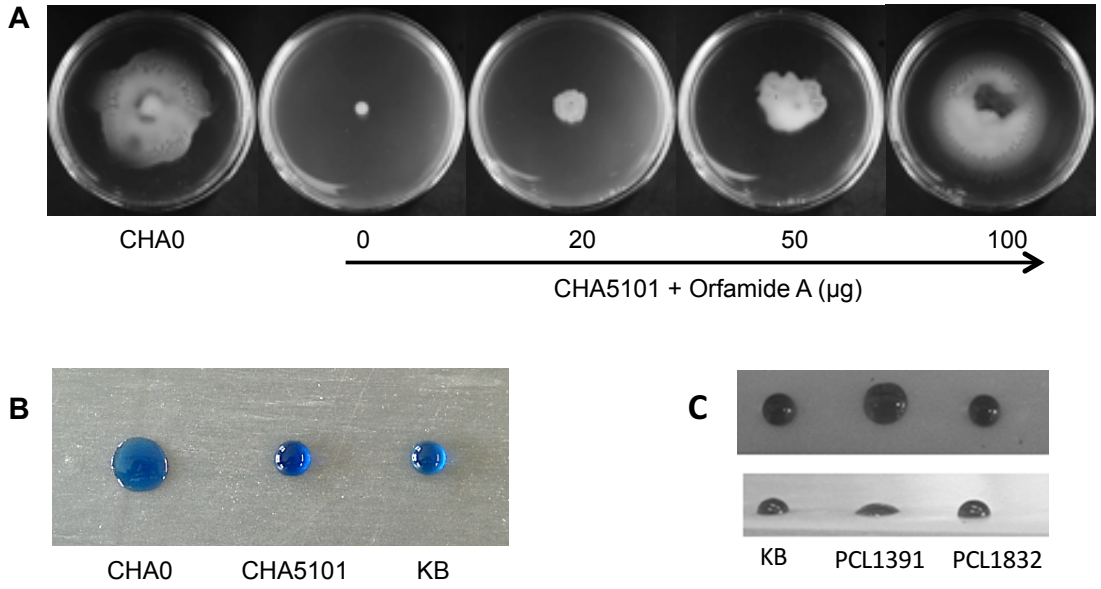
Way J. L. (1984). Cyanide intoxication and its mechanism of antagonism. *Annu Rev Pharmacol Toxicol* 24**:** 451-481.

Wolk C. P., Cai Y., Panoff J. M. (1991). Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. *Proceedings of the National Academy of Sciences of the United States of America* 88**:** 5355-5359.

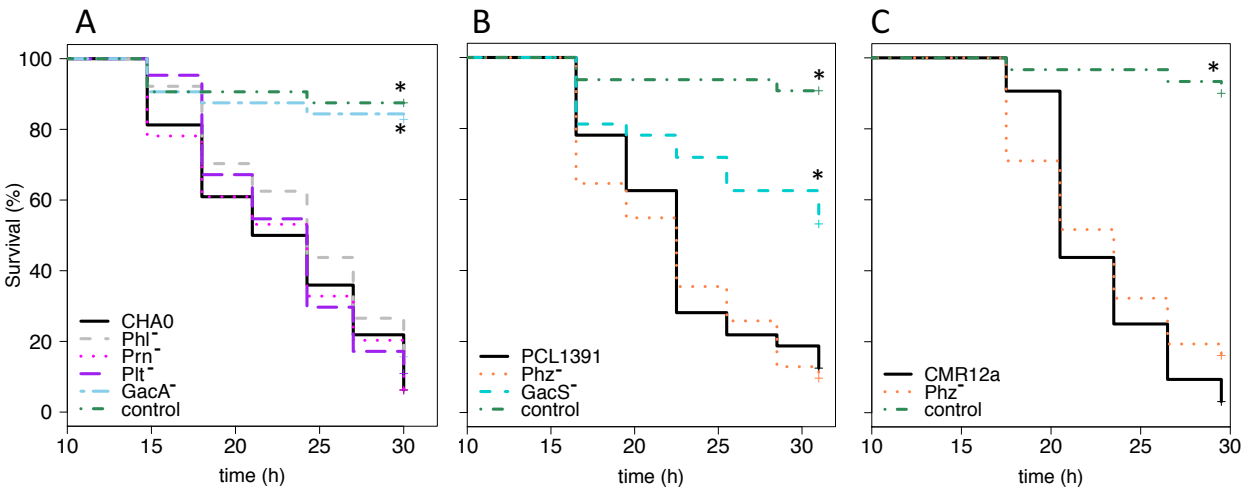
Workentine M. L., Chang L., Ceri H., Turner R. J. (2009). The GacS-GacA two-component regulatory system of *Pseudomonas fluorescens*: a bacterial two-hybrid analysis. *FEMS Microbiol Lett* 292**:** 50-56.

Zuber S., Carruthers F., Keel C., Mattart A., Blumer C., Pessi G. *et al.* (2003). GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Molecular plant-microbe interactions : MPMI* 16**:** 634-644.

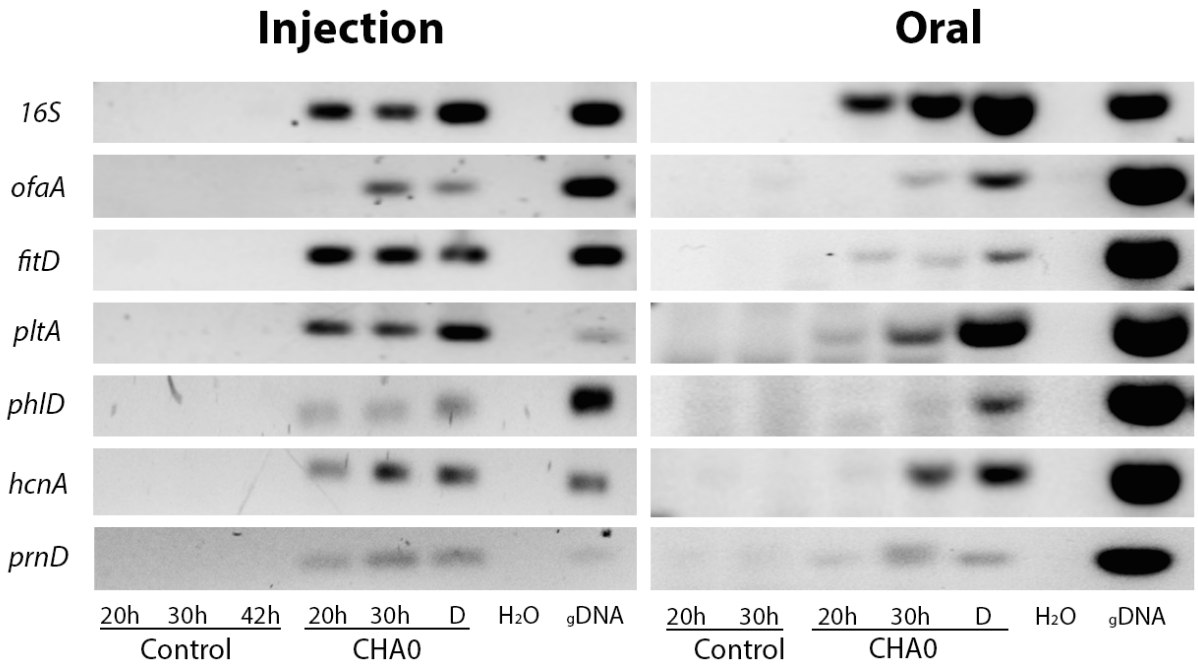
SUPPLEMENTARY INFORMATION



Supplementary Figure S1 Characterization of Clp mutants of *P. protegens* CHA0 and *P. chlororaphis* PCL1391. (A) Surface swarming motility of *P. protegens* CHA0 and its isogenic Δ*ofaABC* mutant CHA5101. 5 µL of washed bacterial cells were dropped to the center of soft agar LB plates (0.6% w/v agar). Pictures were taken after 20 h of incubation at 28 °C. The orfamide mutant CHA5101 was complemented by adding orfamide A of *P. protegens* CHA0 to the medium at the indicated concentrations (µg/plate). **(B, C)** Droplet collapse assay. Supernatants of KB overnight cultures of *P. protegens* CHA0, *P. chlororaphis* PCL1391 and their isogenic mutants CHA5101 und PCL1832 (insertional mutation in *ofaC* homolog) were spotted on parafilm and observed for loss of surface tension. Evan blue solution **(B)** or methylene blue **(C)** were added for photographic purposes and had no influence on the shape of the droplets. Experiments were repeated independently for three times with similar results and representative pictures are shown.



Supplementary Figure S2 Antimicrobial metabolites without significant impact on oral insecticidal activity of selected pseudomonads against *Plutella xylostella*. For *Pseudomonas protegens* strain CHA0 **(A)** 64 larvae were exposed to artificial diet inoculated with 4 x 10^6 bacterial cells. In experiments with strain *Pseudomonas chlororaphis* strain PCL1391 **(B)** and *Pseudomonas* sp. CMR12a **(C)** 32 larvae were exposed to 2 x 10^7 bacterial cells. Treatments that differed significantly from their respective wild type strain (Log-Rank test $p \leq 0.05$, Survival Package in R) are marked with an asterisk. LT_{50} values of the experiments presented above and of a repetition of the experiment are listed in Tables 2 and 3. Each strain was tested at least three times and similar results were obtained. Solid black line, wild-type strain; dashed grey line, mutant deficient 2,4-diacetylphloroglucinol production (Phl$^-$, CHA1241); dotted pink line, mutant deficient for pyrrolnitrin production (Prn$^-$, CHA5091); dashed purple line, mutant deficient pyoluteorin production (Plt$^-$, CHA5092); dash-dot light blue line, mutant deficient for GacA production (GacA$^-$, CHA89); orange dotted lines, mutants deficient for the production of phenazines (Phz$^-$, PCL1113, CMR12a-ΔPhz); dashed turquois line, mutant deficient for GacS production (GacS$^-$, PCL1123); dash-dot green line, 0.9% NaCl solution control.



Supplementary Figure S3 Expression of biosynthetic genes for antimicrobial metabolites of *Pseudomonas protegens* CHA0 during insect infections. *Galleria mellonella* larvae were infected systemically by injection of 2 x 10^3 cells of *Pseudomonas protegens* CHA0 (Injection). Hemocoel was collected 20 h and 30 h post injection (living larvae) and after 42 h when larvae had succumbed to infection. *Plutella xylostella* larvae were infected orally by feeding artificial diet inoculated with 4 x 10^6 cfu (Oral). Larvae were collected after 20 h, 30 h, and when death occurred (D). Total RNA was extracted from hemolymph (*Galleria*) or entire insects (*Plutella* larvae), converted into cDNA, and gene expression for the indicated genes was determined by PCR using specific primers (Table S2). Control larvae were treated with sterile 0.9% NaCl solution. Genomic DNA of CHA0 (gDNA) served as positive control.

Supplementary Table S1 Plasmids and primers used to create deletion mutants

Plasmid or oligonucleotide	Relevant characteristics or sequence (5′ → 3′)	Reference or comment
Plasmids		
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r, Ap^r	Martinez-Garcia and de Lorenzo (2011)
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	Martinez-Garcia and de Lorenzo (2011)
pME8314	pEMG-Δ*prnABCD*; suicide plasmid for the deletion of the *prnABCD* gene cluster (PFLCHA0_c36450 to PFLCHA0_c36480) in CHA0; Km^r	This study
pME8315	pEMG-Δ*pltABCDEFG* suicide plasmid for the deletion of the *pltABCDEFG* gene cluster (PFLCHA0_c28450 to PFLCHA0_c28510) in CHA0; Km^r	This study
pME8322	pEMG-Δ*hcnABC* suicide plasmid for the deletion of the *hcnABC* gene cluster (PFLCHA0_c26420 to PFLCHA0_c26440) in CHA0; Km^r	This study
pME8325	pEMG-Δ*ofaABC*; suicide plasmid for the deletion of the *ofaABC* gene cluster (PFLCHA0_c21860 to PFLCHA0_c21880) in CHA0; Km^r	This study
pME8400	pEMG-Δ*phlACBD* suicide plasmid for the deletion of the *phlACBD* gene cluster (PFLCHA0_c59080 to PFLCHA0_c59110) in CHA0; Km^r	This study
pME11047	pEMG-Δ*hcnABC* suicide plasmid for the deletion of the *hcnABC* gene cluster (PCL1391_2240 to PCL1391_2242) in PCL1391; Km^r	This study
Primers		
ofaABC-1	CGGAATTCCCGATGAACCTGATCCAGTTCT, EcoRI	Deletion of CHA0 *ofaABC*
ofaABC-2	GGGGTACCCAGTTGGTCGAGCCAGATATC, KpnI	Deletion of CHA0 *ofaABC*
ofaABC-3	GGGGTACCAACCACTTCAGCCTGCTCAAGG, KpnI	Deletion of CHA0 *ofaABC*
ofaABC-4	CGGGATCCAGTCACGGTAGCGCTCGTAGAT, BamHI	Deletion of CHA0 *ofaABC*
prn-1	GGAATTCAATTGGCTCAAGGACAGTTGGTTC, EcoRI	Deletion of CHA0 *prnABCD*
prn-2	CCCAAGCTTCATAACACTCCCTGTTTCGAGG , HindIII	Deletion of CHA0 *prnABCD*
prn-3	CCCAAGCTTAAGTACCGTGCGTTCTACCGC, HindIII	Deletion of CHA0 *prnABCD*
prn-4	CGGGATCCGCGAGCGTATCTTTCGAGACG, BamHI	Deletion of CHA0 *prnABCD*
PphlACBD-1	GGGGTACCCCTTAAGAGATTAGATCGTCTG, KpnI	Deletion of CHA0 *phlACBD*
PphlACBD-2	GGAATTCGTCATAGGGATTGGTGCAGGTGC, EcoRI	Deletion of CHA0 *phlACBD*
PphlACBD-3	GGAATTCAACCTCAATCGCGGCGACATCGG, EcoRI	Deletion of CHA0 *phlACBD*
PphlACBD-4	GCTCTAGAGACAATGATGCTGGTGGGGGTG, XbaI	Deletion of CHA0 *phlACBD*
plt-1	GGAATTCAGGTGGGATGCCAAGTAGTCT, EcoRI	Deletion of CHA0 *pltABCDEFG*
plt-2	CCCAAGCTTCATAGACGTACGCTCCTGCA, HindIII	Deletion of CHA0 *pltABCDEFG*
plt-3	CCCAAGCTTGTGTGAGCCGACTATTGGGC, HindIII	Deletion of CHA0 *pltABCDEFG*
plt-4	CGGGATCCGACGGCGAACACACTAAAATCC, BamHI	Deletion of CHA0 *pltABCDEFG*
hcn-1	CGGAATTCAGGCCGTGGAAGAAGCCAAGCA, EcorI	Deletion of CHA0 *hcnABC*
hcn-2	GGGGTACCTATCTGACGCATTGTGGGTTCA, KpnI	Deletion of CHA0 *hcnABC*
hcn-3	GGGGTACCGTGAAGAAACAGCCCGAACCGG, KpnI	Deletion of CHA0 *hcnABC*
hcn-4	CGGGATCCTTTCGGTGTTCAGCACCTTCGA, BamHI	Deletion of CHA0 *hcnABC*
hcn1391-del-1	GGAATTCAAGTCATCGCCAGCCTCGAGGCC, EcoRI	Deletion of PCL1391 *hcnABC*
hcn1391-del-2	CCCAAGCTTTTCGGTATGGCGCATCAGGAA, HindIII	Deletion of PCL1391 *hcnABC*
hcn1391-del-3	CCCAAGCTTTTGCAGGTGGCGTTGGCCTGA, HindIII	Deletion of PCL1391 *hcnABC*
hcn1391-del-4	CGGGATCCGACGACACGGTGCTGATGGATT, BamHI	Deletion of PCL1391 *hcnABC*

Ap^r, ampicillin; Gm^r, gentamicin; Km^r, kanamycin resistance, respectively. Specified restriction sites are underlined.

Supplementary Table S2 Primers used for qualitative gene expression analysis.

Target gene	Primers	Sequence (5'→3')	Annealing temp.	Reference or comment
phlD	PhlD_65F_DEG PhlD_236R_DEG	GGT RTG GAA GAT GAA RAA RTC GCC YRA BAG YGA GCA YTA C	50°C (55°C)	This study
fitD	FitD_66F_DEG FitD_308R_DEG	CTA TCG GGT SCA GTT CAT CA TTC TTG TCG GSA AAC CAC T	60°C	This study
prnD	PrnD_F PrnD_R	TGC ACT TCG CGT TCG AGA C GTT GCG CGT CGT AGA AGT TCT	60°C	Garbeva *et al.* (2004)
Pseudomonas spp., 16s rRNA	Pse435F Pse686R	ACT TTA AGT TGG GAG GAA GGG ACA CAG GAA ATT CCA CCA CCC	60°C	Bergmark *et al.* (2012)
hcnA	HcnA_F HcnA_R	CGG GCT CAA GTT CGT CAT CT AAG TAC ACA TCC ACG CCG TT	60°C	This study
pltA	PltA_F PltA_R	TGA CGT CGA GTT TCT CAG CC GGT CAT CGG CAG GAA GTG AA	60°C	This study
ofaA	OfaA_F OfaA_R	GGC CTG CTC TAT CAC CAC ATG CCT GCC ATT CTT GAA CCG TCA	60°C	This study

References

Bergmark L., Poulsen P. H., Al-Soud W. A., Norman A., Hansen L. H., Sorensen S. J. (2012). Assessment of the specificity of *Burkholderia* and *Pseudomonas* qPCR assays for detection of these genera in soil using 454 pyrosequencing. *FEMS Microbiol Lett* 333**:** 77-84.

Garbeva P., Voesenek K., van Elsas J. D. (2004). Quantitative detection and diversity of the pyrrolnitrin biosynthetic locus in soil under different treatments. *Soil Biology & Biochemistry* 36**:** 1453-1463.

Martinez-Garcia E., de Lorenzo V. (2011). Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Environ Microbiol* 13**:** 2702-2716.

CHAPTER 4

The plant-beneficial root-colonizer *Pseudomonas protegens* CHA0 persists in insects throughout different developmental stages and can be transmitted to new host plants

A version of this thesis chapter is in preparation for publication by

Pascale Flury[1], Pilar Vesga[1], Ana Dominguez-Ferreras[1], Dylan Bär[1], Camille Tinguely[1], Jana Schneider[1], Anja Taddei[1], Cornelia Ullrich[2], Regina Kleespies[2], Christoph Keel[3]* and Monika Maurhofer[1]*

[1] Plant Pathology, Institute of Integrative Biology, ETH Zürich, Zürich, Switzerland
[2] Julius Kühn-Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Biological Control, Darmstadt, Germany
[3] Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland
*corresponding authors

ABSTRACT

The discovery of insecticidal activity in root-colonizing pseudomonads, best-known for their plant-beneficial effects, raised on one hand fundamental questions about the ecological relevance of insects as alternative hosts for these bacteria and on the other hand implied a possible application of this trait in pest control. 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 plant-beneficial and insecticidal bacterium *Pseudomonas protegens* CHA0 from plant to plant by the cabbage root fly, *Delia radicum*. After ingestion by *D. radicum* larvae, CHA0 persisted inside the insect host until the pupal stage, a phenomenon, which was additionally observed in three other insect species. Next, CHA0 was successfully transmitted to a fresh plant host by emerging flies, as indicated by high levels of root colonization in the transmission microcosms. Because *D. radicum* is a major root pest on various cabbage crops, we further assessed the biocontrol potential of CHA0 against this insect species. No effect on insect survival could be detected, but morphological defects observed in parts of adult *D. radicum* indicate a negative impact of *P. protegens* CHA0 on insect development. In summary, this study shows that a plant-beneficial rhizobacterium can be dispersed by insects and suggests that fluorescent pseudomonads can use insects as hosts and vectors.

INTRODUCTION

Every year worldwide crop production is facing major harvest losses due to plant pathogens and insect pests. Chemical pesticides are commonly used and an efficient means in crop protection, but increasing resistance in target pathogens and insect pests as well as the rising attention on their adverse environmental effects demand for alternative strategies. The use of antagonistic organisms to control pest organisms, known as biological control, represents a sustainable approach, since it does not pollute the environment with toxic synthetic compounds and is less susceptible to the development of resistance (Holt and Hochberg, 1997). Nonetheless, the safe introduction of an organism into an agro-ecosystem entails a sound knowledge on its properties—its modes of action, but also its ecological impact.

Soil-borne diseases and insect pests are especially difficult to tackle with chemical pesticides. Therefore, plant-beneficial rhizobacteria with disease suppressive capacity, e.g. belonging to the genera *Pseudomonas* and *Bacillus*, gained increasing attention as potential biocontrol agents, and extensive research on these organisms already resulted in several commercial products (Berg, 2009). An extremely versatile representative of this group of bacteria is *Pseudomonas protegens* CHA0. This strain, which was isolated from the rhizosphere of tobacco (Stutz *et al.*, 1986), efficiently colonizes roots of diverse plant species to densities as high as 10^7 to 10^8 bacteria per g of roots (Keel *et al.*, 1992; Maurhofer *et al.*, 1994b). *P. protegens* CHA0, which produces a distinct set of antimicrobial compounds, exhibits biocontrol effects against several soil-borne pathogens of agronomic importance, such as *Rhizoctonia*, *Pythium*, *Fusarium,* and *Gaeumannomyces* and, in addition, can induce systemic resistance against above ground pathogens (Maurhofer *et al.*, 1994a; Maurhofer *et al.*, 1994b; Maurhofer *et al.*, 1995; Lemanceau *et al.*, 2007).

Although *P. protegens* CHA0 and related strains, phylogenetically belonging to the *Pseudomonas chlororaphis* subgroup (Mulet *et al.*, 2012), are very well adapted to a life in the rhizosphere, their habitat is not restricted to plant roots, since these bacteria can also infect and kill insect larvae (Péchy-Tarr *et al.*, 2008; Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2015; Flury *et al.*, 2016; Rangel *et al.*, 2016). Strains of the *P. chlororaphis* subgroup were found to exhibit oral activity against larvae of several lepidopteran species as well as against *Drosophila melanogaster* and against *Diabrotica virgifera*

virgifera (Olcott *et al.*, 2010; Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2013; Flury *et al.*, 2016; Rangel *et al.*, 2016; Schellenberger *et al.*, 2016). Several factors have been identified to contribute to their insecticidal activity: the Fit toxin, antimicrobial metabolites (rhizoxin, cyclic lipopeptides, hydrogen cyanide), secreted enzymes (chitinase C, phospholipase C), lipopolysaccharide O antigen, the insecticidal protein IPD072Aa, and the global regulatory system GacS/GacA (Péchy-Tarr *et al.*, 2008; Devi and Kothamasi, 2009; Olcott *et al.*, 2010; Jang *et al.*, 2013; Ruffner *et al.*, 2013; Ruffner *et al.*, 2015; Flury *et al.*, 2016; Keel, 2016; Kupferschmied *et al.*, 2016; Loper *et al.*, 2016; Schellenberger *et al.*, 2016)(Chapter 3). In-depth studies on the Fit toxin revealed that this insecticidal factor is specifically produced in insects, but not on plant roots (Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014). Accordingly, the bacteria seem to sense their environment and regulate the production of specific compounds depending on the specific needs of the encountered habitat. Although there is a growing body of evidence that insects represent an alternative host for *P. protegens* CHA0, the ecology of its insect-associated lifestyle is still elusive, and insecticidal activity of *P. protegens* CHA0 has yet been investigated only in model insects feeding on leaves. Since *P. protegens* CHA0 and related strains were isolated from roots, it is evident that activity against a root-feeding insect would be of greater ecological relevance as well as of interest for potential applications in pest control.

Rhizobacteria are limited in their inherent dispersal abilities and may largely depend on passive transport, such as groundwater flows to overcome large distances. Alternatively, dispersal by means of a vector is a plausible manner of conquering new habitats. The discovery that several plant-beneficial pseudomonads exhibit specific adaptions to life in insects (Kupferschmied *et al.*, 2013; Keel, 2016) raises the hypothesis that insects might not only represent alternative hosts for these bacteria, but also vectors to reach a new plant host.

Insect-mediated dispersal has been described for several plant-pathogenic bacteria (Nadarasah and Stavrinides, 2011), but data on transmission of beneficial rhizobacteria to a new host plant is very 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* (Snyder *et al.*, 1998; Snyder *et al.*, 1999). However, *P. chlororaphis* L11 moves into the foliage

(Lamb *et al.*, 1996) and in most experiments insect vectors were feeding on L11-infested foliage prior to transmission to the roots of a new host plant. *P. protegens* CHA0, in contrast, was not found on above-ground plant parts even if roots were highly colonized (Maurhofer *et al.*, 1998; Iavicoli *et al.*, 2003). 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 adult stage, moving to new host plants, 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.

The main aim of this study was to investigate for the first time whether *P. protegens* CHA0 exhibits biocontrol activity against a root-feeding insect pest and whether a root-feeding insect could serve as a vector for dispersal of this plant-beneficial rhizobacterium. In this regard, it was also of interest whether *P. protegens* CHA0 is able to persist in insect hosts throughout different life stages. Therefore, a test system with larvae of *Delia radicum*, a major root pest on brassicaceous plants, which were feeding on cauliflower roots colonized by *P. protegens* CHA0, was developed. Larvae of *D. radicum* pupate in the soil and emerging adults fly to a new host plant to deposit eggs. Although no significant difference in insect survival was detected between control and CHA0 treatment, the observation that some flies emerging from CHA0 treated larvae exhibited strong morphological defects gives first indications that the bacterial treatment affects *D. radicum* fitness. Furthermore, *P. protegens* CHA0 was indeed able to persist in *D. radicum* throughout different life stages and was transmitted to new host plants, which provides the first evidence for the possibility of insect-mediated dispersal of *P. protegens* CHA0. Transstadial transmission of *P. protegens* CHA0 seems to be a rather general phenomenon as it was observed in three further insect species, tested in this study. Finally, we developed a microscopy-based method, which allows to examine the bacterial localization inside insect hosts and to assess potential impacts on insect tissues, and we present first observations on the course of infection.

MATERIALS AND METHODS

Bacterial cultures

Bacteria from the long-term storage at -80°C were grown for two days on King's B (KB) agar (King *et al.*, 1954), which was supplemented with chloramphenicol (13 µg ml^{-1}), cycloheximide (100 µg ml^{-1}) and ampicillin (40 µg ml^{-1})(KB^{3+}) for cultivation of *P. protegens* CHA0. To grow *P. protegens* CHA0-*gfp2* (CHA0::attTn*7*-*gfp2*, Gm^r) and *P. protegens* CHA1176 (CHA1163::*att*Tn*7*-*gfp2*, Gm^r; CHA1163 is a CHA0 derivative expressing a C-terminal FitD–mCherry fusion protein)(Péchy-Tarr *et al.*, 2013) ampicillin was replaced by gentamycin (10 µg ml^{-1})(KB^{CCG}). Bacteria grown on plates were used to inoculate 10 ml LB (supplemented with 10 µg ml^{-1} gentamycin for *P. protegens* CHA0-*gfp2* and *P. protegens* CHA1176). LB cultures were grown overnight at 24°C, 180 rpm. For experiments with *Plutella xylostella*, *Pieris brassicae*, and *Otiorhynchus sulcatus* LB cultures were washed twice in sterile 0.9% NaCl solution (H_2O for *O. sulcatus*), OD_{600} was measured, and suspensions diluted to the desired concentration before adding to insect diets or roots. For experiments with *D. radicum* 200 µl of LB cultures were used to inoculate KB plates supplemented with gentamycin (10 µg ml^{-1}). After one day, bacteria were scrapped off the plates, suspended, washed twice in sterile ddH_2O, and OD_{600} was adjusted to the desired concentration.

Biocontrol experiment with *D. radicum*

To start our own *D. radicum* rearing, pupae were kindly provided by Anne-Marie Cortesero (University of Rennes, France) and Jaka Razinger (ZHAW, Wädenswil, Switzerland). *D. radicum* was reared as described by Razinger *et al.* (2014), but larvae were fed on turnip cabbage instead of rutabaga. 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 ddH_2O, subsequently placed for 30 min in 4% bleach and again thoroughly washed with sterile ddH_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 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 (Keel *et al.*, 1989). Each pot was

amended with 10 ml of bacterial suspension (CHA0-*gfp2*, OD_{600} = 0.45) 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, 15 klux), 8-h night cycle (18°C) and a relative humidity of 80%. For application of *D. 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. Four weeks later, plant shoots were weighed and root systems were washed on a sieve to collect pupae as well as non-pupated larvae. Root colonization was assessed in all control pots as well as in four pots per tray of the *P. protegens* CHA0-*gfp2* treated plants. To this end about 300 mg root sub-sample was placed in an Eppendorf tube containing 0.9% NaCl and incubated at 3°C over-night. Next, roots were shaken for 30 min at 1400 rpm on an Eppendorf thermomixer compact at 4 °C. Serial dilutions were plated onto KB^{CCGA} (KB^{CCG} additionally supplemented with ampicillin (40 µg ml^{-1})), plates were incubated at 27°C and after 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. At the time of root harvest all larvae which had not yet pupated were extracted for bacteria monitoring. All pupae emerging from one plant were photographed together to assess pupal size, which was measured by means of an ImageJ macro measuring number and mean size of pupae. Ten to twelve pupae per treatment were in the following extracted to assess bacterial colonization, a part of the pupae was used for the transmission experiments (see below) and the remaining pupae (44-56 per treatment) were observed to determine hatching rates. To assess bacterial colonization rates, insects were homogenized in 1 ml sterile 0.9% NaCl solution with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). Larvae and pupae were surface sterilized (20 s 0.05% SDS, 20 s 70% Ethanol, 20s sterile ddH_2O) prior to homogenization. The resulting suspensions were serially diluted and plated onto KB^{CCGA}. Colony forming units were checked for expression of GFP as described above. The experiment was conducted twice.

Transmission experiment with *D. 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 filled with 3 cm of autoclaved sand-vermiculite mix supplemented with 35 ml of Knop solution (Keel *et al.*, 1989). Four pre-germinated seeds were planted per beaker and grown for four weeks in a growth chamber with a 16-h day (20°C, 15 klux), 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 (Razinger *et al.*, 2014). Three to four pupae that had emerged from control or *P. protegens* CHA0-*gfp2* treatments (see above) were added per beaker in small sterile Erlenmeyer flasks (experiment A) or sterile lids of Eppendorf tubes (experiment B). In experiment A, 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 B, 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. The experiment was conducted twice.

Survival and colonization experiments with *P. xylostella*

The experiments to assess the persistence of CHA0-*gfp2* in *P. xylostella* at different insect life-stages were set up as described in chapter 3. Eggs of *P. xylostella* were obtained from Syngenta Crop Protection AG (Stein, Switzerland). 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 or 0.01. Experiments 1 and 2 were set up with 32 larvae per treatment and 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. Larvae, pupae, and adults were surface sterilized for 20 s in 70% ethanol and rinsed in sterile ddH_2O prior to homogenization in 0.9% NaCl with a Polytron PT-DA 2112 blender (Kinematica, Littau,

Switzerland). Resulting suspensions were plated on KB^{CCGA} and growing colonies were checked for expression of GFP.

Experiments with *O. 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 suspension of *P. protegens* CHA0 (OD_{600} = 0.0125) or in water for control treatments and in the following planted in pots containing potting soil (TREF Go PP 7000 plant substrate, GVZ Rossat AG, Switzerland). Ten last-instar larvae 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. 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) 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^{3+}. The identity of growing colonies was checked as described by Ruffner (2013) with a colony PCR using primers that specifically amplify *P. protegens* CHA0 (Von Felten *et al.*, 2010) and by sequencing a part of the *16s rRNA* gene.

Colonization experiments with *P. brassicae*

P. brassicae eggs were kindly provided by the Environmental Systems Sciences group of ETH Zurich (Switzerland). Larvae were reared at 25°C, 60% relative humidity and a 16-h day, 8-h night cycle. For the experiment, 4th instar larvae were kept individually in Petri dishes lined with a moisturized filter paper and were fed with a pellet of artificial diet inoculated with 10 µl of suspension of *P. protegens* CHA0-*gfp2* cells at an OD_{600} of 20 or sterile 0.9% NaCl (control). The diet was modified from David and Gardiner (1965) and consists of casein, 12.6 g; D-sucrose, 12.6 g; corn meal, 10.8 g; α-cellulose, 1.6 g; choline chloride 10%, 3.6 ml; linseed oil, 2 ml; horseradish powder, 30 g; Bacto-agar, 9 g; L-ascorbic acid, 3g; vitamin stock solution, 1.2 ml (Supplementary Table S1); salt mix solution, 65 ml (Supplementary Table S2); dissolved in 600 ml of distilled water. 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), in which they were fed with white cabbage until they pupated. Thirty larvae per treatment were used for monitoring mortality. Larvae and pupae were considered dead when they did not react to poking. Ten to eighteen individuals (alive and dead) of each developmental stage (larva, pupa and butterfly) were collected from both treatments, surface sterilized for 20 s in 70% Ethanol and rinsed in sterile ddH_2O prior to homogenization in 0.9% NaCl with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). Resulting suspension were plated on KB^{CCG} and GFP-expression of growing colonies was verified under the microscope at 480/440 nm. The experiment was conducted twice.

Microscopy

For microscopy, twelve *P. xylostella* larvae were kept together in a Petri dish, lined with a wetted filter paper and four pellets of artificial diet. For the bacterial treatments, *P. protegens* CHA0 and *P. protegens* CHA1176, 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 expressing strain *P. protegens* CHA1176 was used instead of *P. protegens* 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.* (2013). Sections were then incubated in mouse anti-GFP (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 four hours at room temperature. Sections were examined with a Leitz Aristoplan microscope (Leica, Wetzlar, Germany) and images captured with an SIS color view II camera (Soft Imaging System GmbH, Münster, Germany).

Statistics

Data analysis was performed in RStudio version 0.98.1017 (http://www.rstudio.com) using R version 3.1.2. To test for significant differences between the control and the *P. protegens* CHA0-*gfp2* treatment, data were tested for normal distribution (Shapiro-Wilk test) and according to the result a Student's t test or a Mann-Whitney *U* test was performed.

RESULTS

D. radicum larvae feeding on roots colonized by *P. protegens* CHA0 are not impaired in survival

In order to answer our three research questions whether *P. protegens* CHA0 (i) exhibits biocontrol activity against a root pest (ii) is able to persist throughout different life-stages in the insect host and (iii) can be transferred by the insect to a new host-plant, we developed a pot experiment with cauliflower plants, inoculated with or without *P. protegens* CHA0-*gfp2,* to which larvae of the root pest *D. radicum* were added and let develop until pupation. The GFP expressing variant of *P. protegens* CHA0 allowed the identification of the bacteria later after re-isolation from roots and insects. Five freshly hatched larvae were placed in immediate vicinity to the plant stem onto the substrate, into which they burrowed to start feeding on the plant roots. After four weeks, pupae emerging from CHA0-*gfp2* treated roots as well as control roots were collected. No significant differences in pupation rate and pupal size could be detected between control and CHA0-*gfp2* treatment in both repetitions of the experiments (Figure 1A, Supplementary Figure S1C, D and Supplementary Figure S2A). Furthermore, no reduction in the number of flies hatching from pupae in the CHA0-*gfp2* treatment compared to control pupae was observed (Figure 1A, Supplementary Figure S2A). On cauliflower roots, *P. protegens* CHA0-*gfp2* developed on average population sizes of 6.51 ± 0.59 and 5.92 ± 0.56 $\log_{10}$ cfu per g of roots in experiment A and B, respectively. Shoot weights from plants inoculated with *P. protegens* CHA0-*gfp2* did not significantly differ from those of control plants (Supplementary Figure S1A, B).

P. protegens CHA0 ingested by *D. radicum* larvae can cause morphological defects

P. protegens CHA0-*gfp2* seemed not to affect *D. radicum* survival, but the bacterial treatment still might impair insect fitness. About 10% of flies that emerged from CHA0-*gfp2* treated roots showed developmental defects, which was not observed in control flies (Figure 1C, Figure 2). Most obvious were the malformations of the wings (Figure 1D) and as a consequence affected adults were unable to fly, which in nature would prevent them from reproduction. Morphological defects in the CHA0-*gfp2* treatment were observed in both experiments, but were only quantified in experiment A.

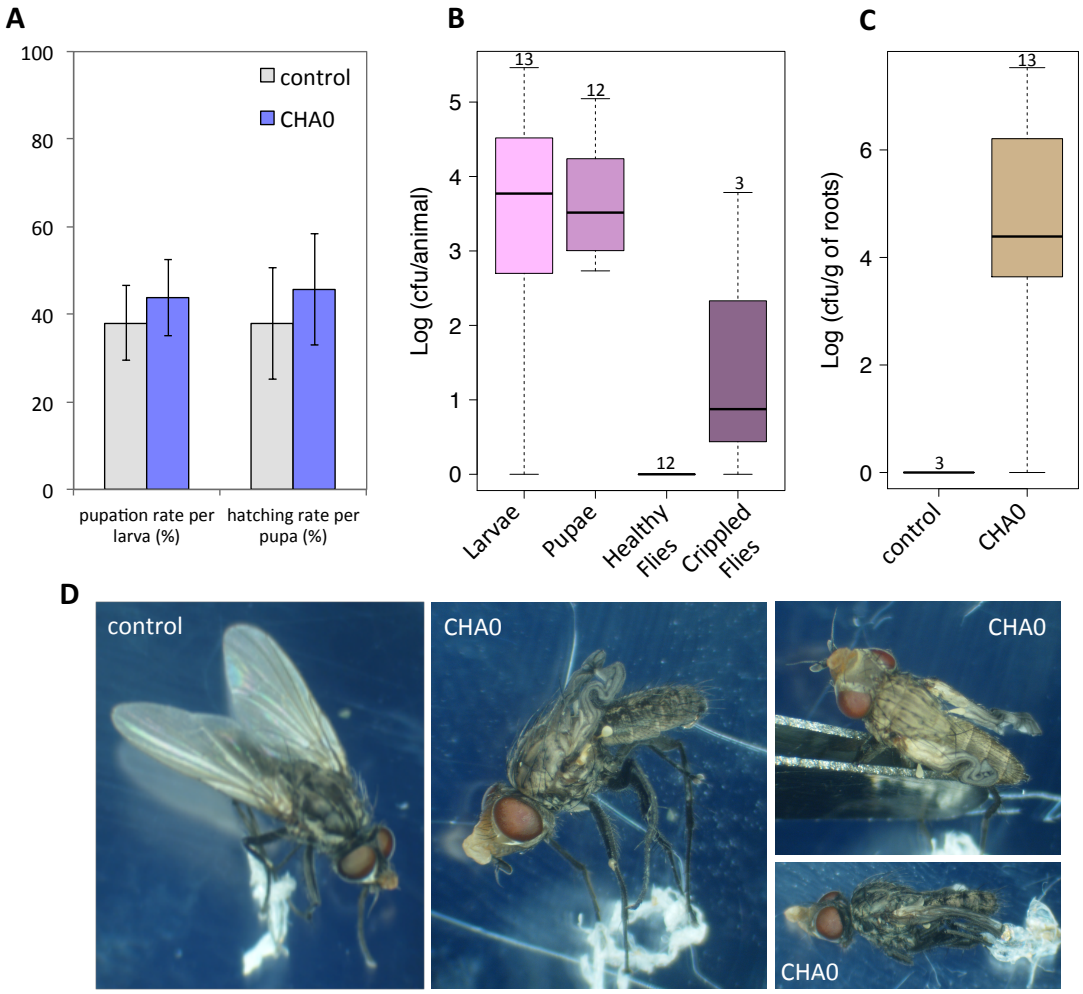


Figure 1 ***Pseudomonas protegens*** **CHA0-*gfp2* does not affect survival of *Delia radicum*, but it persists throughout different life stages and can be dispersed to new host plants by adult flies.** The roots of cauliflower plants (four trays per treatment, each containing twelve pots) were either inoculated with *P. protegens* CHA0-*gfp2* (CHA0) or mock (control). Five freshly hatched *D. radicum* larvae were added per pot. After 3 weeks, emerging pupae as well as remaining larvae were collected. **(A)** Pupation rate and hatching rate of *D. radicum* did not significantly differ between control and *P. protegens* CHA0-*gfp2* treatment ($p < 0.05$; Mann-Whitney *U* test). Error bars depict standard deviations of the means of three to four replicate trays. A repetition of the experiment is depicted in Supplementary Figure S2A. **(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*. Insects were homogenized in 0.9% NaCl and bacterial numbers assessed by plating serial dilutions on selective medium. Larvae and pupae were surface sterilized prior to homogenization. No *P. protegens* CHA0-*gfp2* was detected in individuals emerging from the control treatment (data not shown). A repetition of the experiment is depicted in Supplementary Figure S2B. **(C)** Root colonization of rapeseed plantlets in transmission microcosms. Pupae emerging from cauliflower control and CHA0 treatments were placed into closed sterile systems (3-4 pupae per system) containing rapeseed plantlets grown in a sand-vermiculite mix. To prevent transfer of bacteria by the pupae, they were kept separately from plants and substrate. Emerging flies flew around inside these transmission microcosms and in some cases also laid eggs. Nine days after first flies started to hatch, root systems were collected and bacterial colonization was assessed by dilution plating. In the CHA0 treatment, in twelve out of thirteen systems, roots became colonized with *P. protegens* CHA0-*gfp2*. A repetition of the experiment is depicted in Supplementary Figure S3. **(B, C)** Identities of growing colonies were verified by checking for GFP expression using fluorescence microscopy. Numbers above boxes indicate sample size. **(D)** 10% of flies emerging from the CHA0 treatment showed morphological defects affecting mainly the wings. No morphological defects were observed in control flies.

Transstadial transmission of *P. protegens* CHA0 in *D. radicum*

It is reported that certain strains of the *P. fluorescens* group that are not causing fatal infections are still able to persist inside *Spodoptera littoralis* larvae (Flury *et al.*, 2016). We were wondering whether, although no effect on larval survival was observed, *P. protegens* CHA0-*gfp2* was able to colonize *D. radicum* larvae, and whether the bacteria would persist inside the insect throughout different life stages. Therefore, we extracted larvae, pupae, and adults and assessed bacterial numbers by dilution-plating of extracts. In both repetitions of the experiment, *D. radicum* larvae and pupae were found to be colonized by *P. protegens* CHA0-*gfp2* (Figure 1B, Figure 2 and Supplementary Figure S2B). The bacteria were detected in all extracted pupae of both experiments as well as in eleven out of thirteen larvae of experiment A (Figure 1B) and in all larvae of experiment B (Supplementary Figure S2B). Colonization levels of CHA0-*gfp2* were on average at 3.9 ± 1.0 $\log_{10}$ cfu per larva and 3.5 ± 0.8 $\log_{10}$ cfu per pupa in experiment A and at 3.0 ± 0.6 $\log_{10}$ cfu per larva and 3.9 ± 0.8 $\log_{10}$ cfu per pupa in experiment B. Since insects were surface sterilized before extraction, we believe that the bacteria were indeed inside the insect. No *P. protegens CHA0-gfp2* was detected in insects from the control treatment.

In contrast to results on larvae and pupae, CHA0-*gfp2* was not generally detected in adult flies, but only in those with morphological defects (Figure 1B and Supplementary Figure S2B). Two out of three crippled flies, extracted in experiment A, were colonized by *P. protegens* CHA0-*gfp2* (Figure 1B, Figure 2). Hence, we provide first evidence that *P. protegens* CHA0-*gfp2* can be transstadially transmitted not only to the pupal stage, but, in certain cases, even to the adult stage and that this seems to affect insect development.

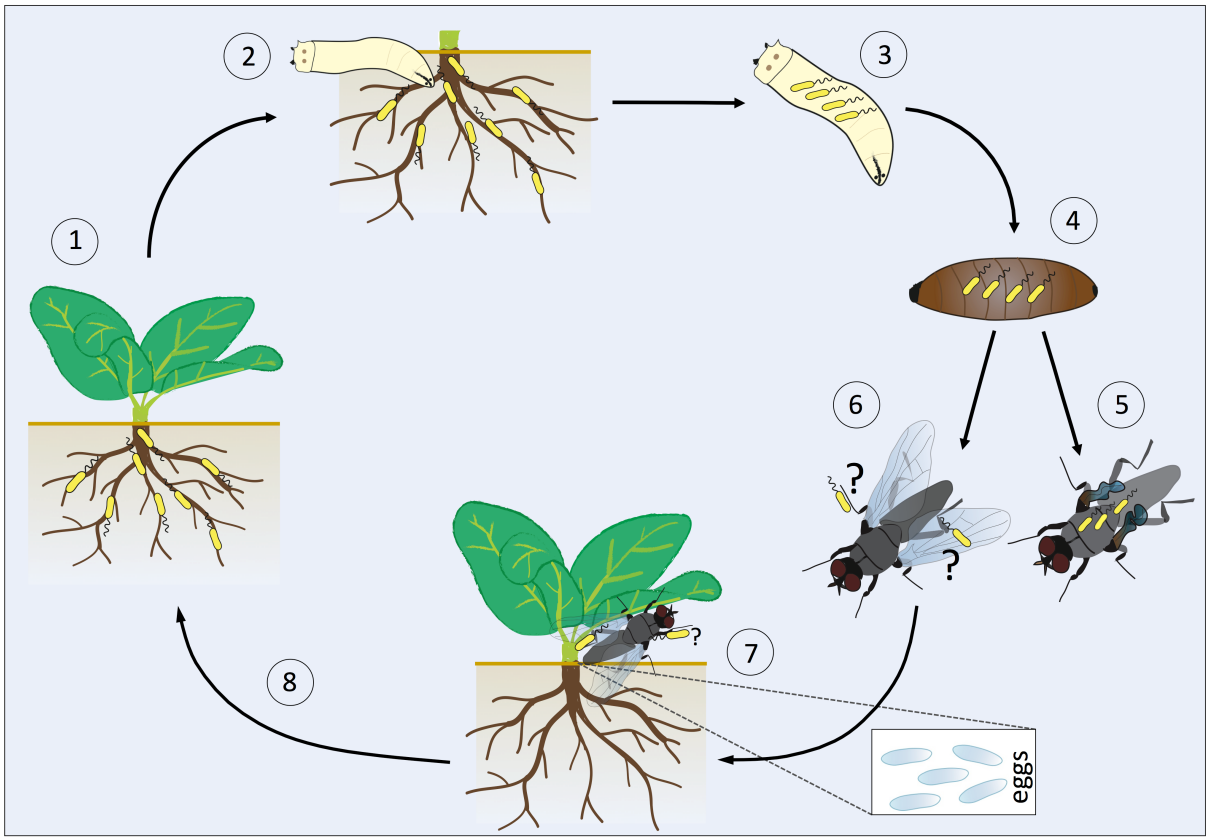


Figure 2 ***Pseudomonas protegens*** **CHA0, taken up by root-feeding** ***Delia radicum*** **larvae, persist in the insect throughout different life stages and is dispersed to new host plants.** Roots of cauliflower plants were inoculated with *P. protegens* CHA0-*gfp2* **(1)** and freshly hatched larvae of *D. radicum* were added to feed on the colonized roots **(2)**. CHA0-*gfp2* was ingested by the larvae **(3)** and found to persist inside the insect also in the pupal stage **(4)** (Figure 1B). 10% of flies emerging from pupae showed developmental defects **(5)** which affected mainly the wings (Figure 1D), and in these individuals *P. protegens* CHA0-*gfp2* could be detected (Figure 1B). 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)**. Although our method failed to detect CHA0 in healthy looking flies, these were able to transmit the bacterium to a new host plant resulting in high bacterial colonization **(8)** (Figure 1C).

P. protegens CHA0 can be dispersed by the insect to a new host plant

To assess, whether *D. radicum* 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 *P. protegens* CHA0-*gfp2* treatments in the cauliflower experiments were transferred into closed plastic beakers 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 hatch, root systems were collected and bacterial colonization was assessed by dilution plating. In experiment A, the roots of twelve out of thirteen transmission microcosms, which had been exposed to flies emerging from the *P. protegens* CHA0-*gfp2* treatment, were indeed colonized by *P. protegens* CHA0-*gfp2* (Figure 1C). Similarly, root colonization by *P. protegens* CHA0-*gfp2* was detected in eight out of nine samples in experiment B (Supplementary Figure S3). In both experiments no CHA0-*gfp2* could be detected on roots from transmission microcosms that had been exposed to flies from the control treatment (Figure 1C, Supplementary Figure S3). In microcosms with successful *P. protegens* CHA0-*gfp2* transmission, colonization rates were on average 5.0 ± 1.8 $\log_{10}$ cfu per g of roots and 4.3 ± 1.1 $\log_{10}$ cfu per g of roots in experiment A and B, respectively. Thus, *D. radicum* flies were able to transmit *P. protegens* CHA0-*gfp2* to a new host plant (Figure 2), although we did not detect any bacteria in healthy flies.

Transstadial transmission of *P. protegens* CHA0, a phenomenon observed in different insect species

The phenomenon of *P. protegens* CHA0 persisting throughout different life stages observed in *D. radicum*, was studied in more detail in further insect species: the root-feeder *Otiorhynchus sulcatus* and the leaf-feeders *Plutella xylostella* and *Pieris brassicae*.

P. protegens CHA0 is highly virulent towards larvae of the diamondback moth, *P. xylostella*. Larvae fed on artificial diet inoculated with 10 µl of OD_{600} = 0.1 of *P. protegens* CHA0-*gfp2* surrendered infection within a few days. Almost 80% of larvae did not survive until pupation and the rest commonly died in the pupal stage (Figure 3A). We assessed colonization rates by *P. protegens* CHA0-*gfp2* in alive and dead larvae and pupae (Figure 3B). 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. While of the living larvae and pupae a few individuals were free from *P. protegens* CHA0-*gfp2*, all dead individuals were highly colonized. 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 at the larval stage anymore (Figure 3A). In these infections *P. protegens* CHA0-*gfp2* was only detected in six out of fifteen larvae and at very low numbers (Figure 3B).

However, colonization rates increased at the pupal and the adult stage (Figure 3B). Generally, adult emergence was very low, also in control treatments (Figure 3A). This is, because the *P. xylostella* feeding assay is optimized for fast killing of larvae upon feeding on *P. protegens* CHA0 and not for long term survival of the insects. Therefore, low levels of adult emergence are probably due to suboptimal diet and husbandry conditions. Nevertheless, the consistent detection of *P. protegens* CHA0-*gfp2* in all extracted imagines indicates transstadial transmission in *P. xylostella* from the larval via the pupal to the adult stage.

A similar observation was made in experiments with the black vine weevil, *O. sulcatus*. Larvae of *O. sulcatus* feed on roots and are often responsible for considerable damage on ornamentals. In pot experiments, roots of strawberry plants were inoculated with *P. protegens* CHA0 and larvae of *O. sulcatus* were added. In order to check for transstadial transmission of *P. protegens* CHA0, emerging pupae and adults were surface sterilized, homogenized and the resulting suspensions plated onto selective medium. Instead of using a GFP expressing variant of CHA0, the identity of growing colonies was checked by strain-specific colony PCR or sequencing of the *16S rRNA* gene. *P. protegens* CHA0 did not affect larval survival, but it was detected in two thirds (experiment A and B) of the pupae and in two thirds (experiment A), respectively all (experiment B) adults of *O. sulcatus*, but bacterial numbers were not quantified (data not shown).

In contrast to results with *P. xylostella* and *O. sulcatus*, *P. protegens* CHA0-*gfp2* was only found until the pupal stage upon oral infections of *P. brassicae* larvae (Figure 3C). Fourth instar larvae were fed with artificial diet inoculated with *P. protegens* CHA0-*gfp2.* Extraction of larvae, pupae and adults revealed presence of the bacteria 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 8 $\log_{10}$ cfu per insect (Figure 3C). However, in living pupae, *P. protegens* CHA0-*gfp2* was only found exceptionally and none of the twenty-nine extracted butterflies harbored the bacteria (Figure 3C). This indicates that in most cases *P. brassicae* larvae either surrender to an infection with *P. protegens* CHA0-*gfp2* or they are able to overcome the disease and eradicate the bacteria until or during pupation. Nevertheless, under certain circumstances *P. protegens* CHA0-*gfp2* might survive until the pupal stage and even cause mortality, since several dead pupae showed considerable numbers of *P. protegens* CHA0-*gfp2* (Figure 3C).

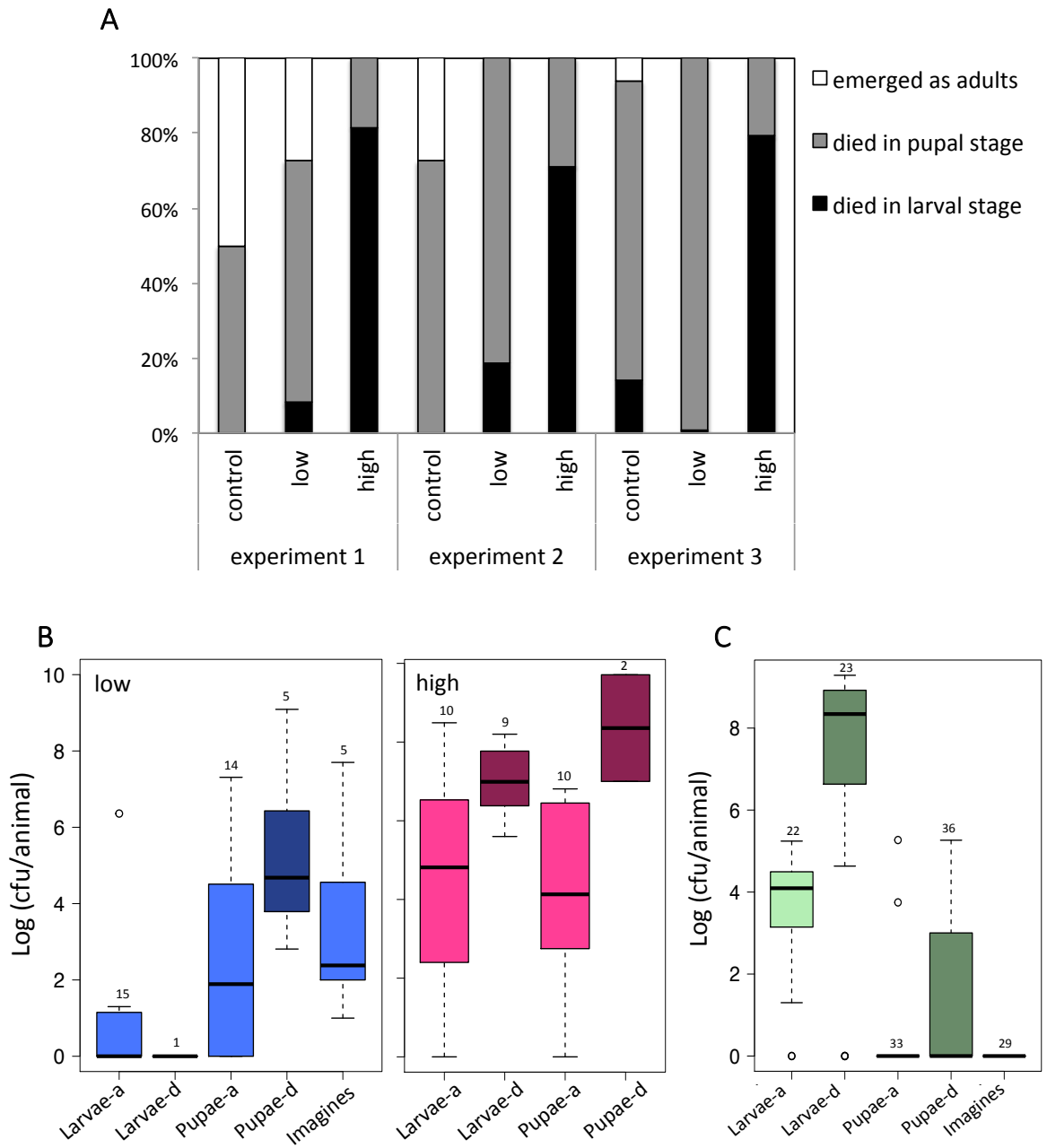


Figure 3 Infection of *Plutella xylostella* and *Pieris brassicae* by *Pseudomonas protegens* CHA0-*gfp2*. (A, B) One-week-old *P. xylostella* larvae were exposed to artificial diet inoculated with a low dosage (low, 10 µl of OD_{600} = 0.01) or a high dosage (high, 10 µl of OD_{600} = 0.1) of *P. protegens* CHA0-*gfp2* or 0.9% NaCl (control). **(A)** Fraction of *P. xylostella* larvae dying at larval or pupal stage or emerging as adults. Three repetitions of the experiment are depicted. **(B)** Colonization of *P. xylostella* by *P. protegens* CHA0-*gfp2*. Data are pooled from all three experiments (low) and from experiment 2 and 3 (high). **(C)** Colonization of *P. brassicae* by *P. protegens* CHA0-*gfp2*. Fourth instar larvae were fed on artificial diet containing 10 µl of a bacterial suspension of OD_{600} = 20. Data are pooled from two experiments. No effect on larval survival was observed in these experiments (data not shown). **(B, C)** In case of larvae and pupae, live (a) as well as dead (d) individuals were extracted. Insects were surface sterilized, homogenized and the resulting homogenate serially diluted and plated onto selective medium. Identity of growing colonies was verified by checking for GFP expression using fluorescence microscopy. Numbers above boxes indicate sample size. No *P. protegens* CHA0-*gfp2* was detected in control insects (data not shown).

Monitoring of *Pseudomonas* infections in *P. xylostella* larvae by microscopy

Although several virulence factors of *P. protegens* CHA0 have been identified (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013; Flury *et al.*, 2016; Keel, 2016; Kupferschmied *et al.*, 2016; Loper *et al.*, 2016; Rangel *et al.*, 2016)(Chapter 3), almost nothing is known about the process of infection upon oral uptake of the bacteria. At one point, massive amounts of bacteria can detected in the hemolymph, but where and how they enter the hemolymph remains speculative (Kupferschmied *et al.*, 2013). In the present work, it was intended to track *P. protegens* CHA0 on its way from the digestive system to the insect hemolymph and in parallel to examine potential histopathological consequences in the insect host. To this end, a microscopy method was developed on histological sections of *P. xylostella* larvae, a species, which is not only highly susceptible to *P. protegens* CHA0, but also small in size. This allowed good penetration of the fixative into the entire larva and reduces the amount of work needed for sectioning and staining. To obtain optimal conditions for histopathological observations and the possibility to track the bacteria within the same sample, it was decided to cut infected insects longitudinally into thin sections of 6 µm and to stain consecutive sections in two different ways. In order to study insect histology, a classical staining with Heidenhain's iron hematoxylin was conducted, whereas, in order to track the bacteria, epifluorescence microscopy on a GFP-expressing variant of *P. protegens* CHA0 (CHA1176) was used. This strain, apart from constitutively expressing GFP, also expresses a C-terminal FitD-mCherry fusion protein. Unfortunately, the tissue fixation with Duboscq-Brazil's alcoholic Bouin's, an optimal fixative for histopathology, destroys the intrinsic fluorescence of the fluorescent proteins. Therefore, *P. protegens* CHA1176 had to be visualized indirectly by means of anti-GFP antibodies.

P. xylostella larvae were fed on artificial diet inoculated with bacterial suspension and harvested at different time points over four days for microscopic analysis. A first set of larvae coming from two independent experiments has been analyzed and representative pictures are shown in Figures 4-6. However, observations are still preliminary and larger sample sizes have to be examined to draw a conclusive picture on the infection process. Nevertheless, the yet conducted investigations gave a first insight into colonization of *P. xylostella* larvae by *P. protegens* CHA0.

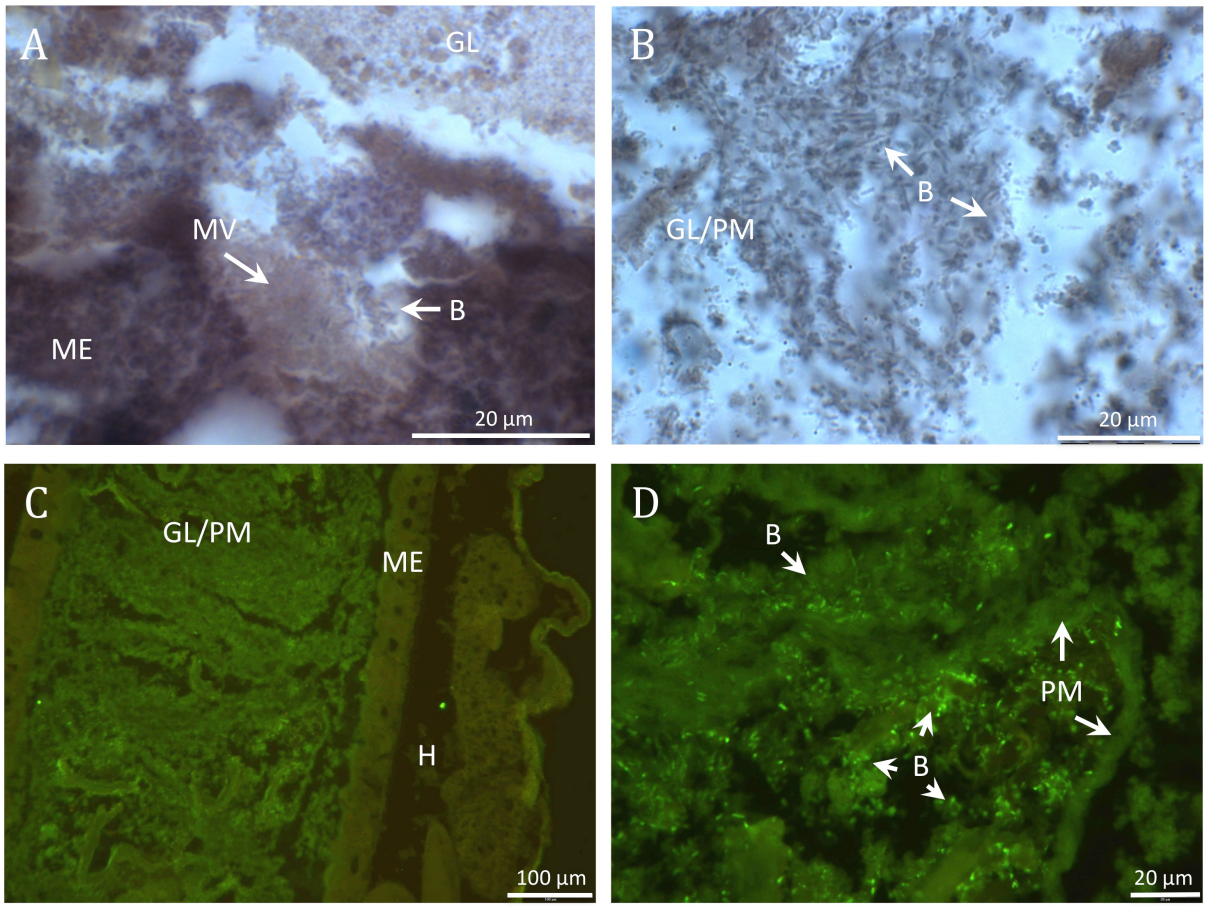
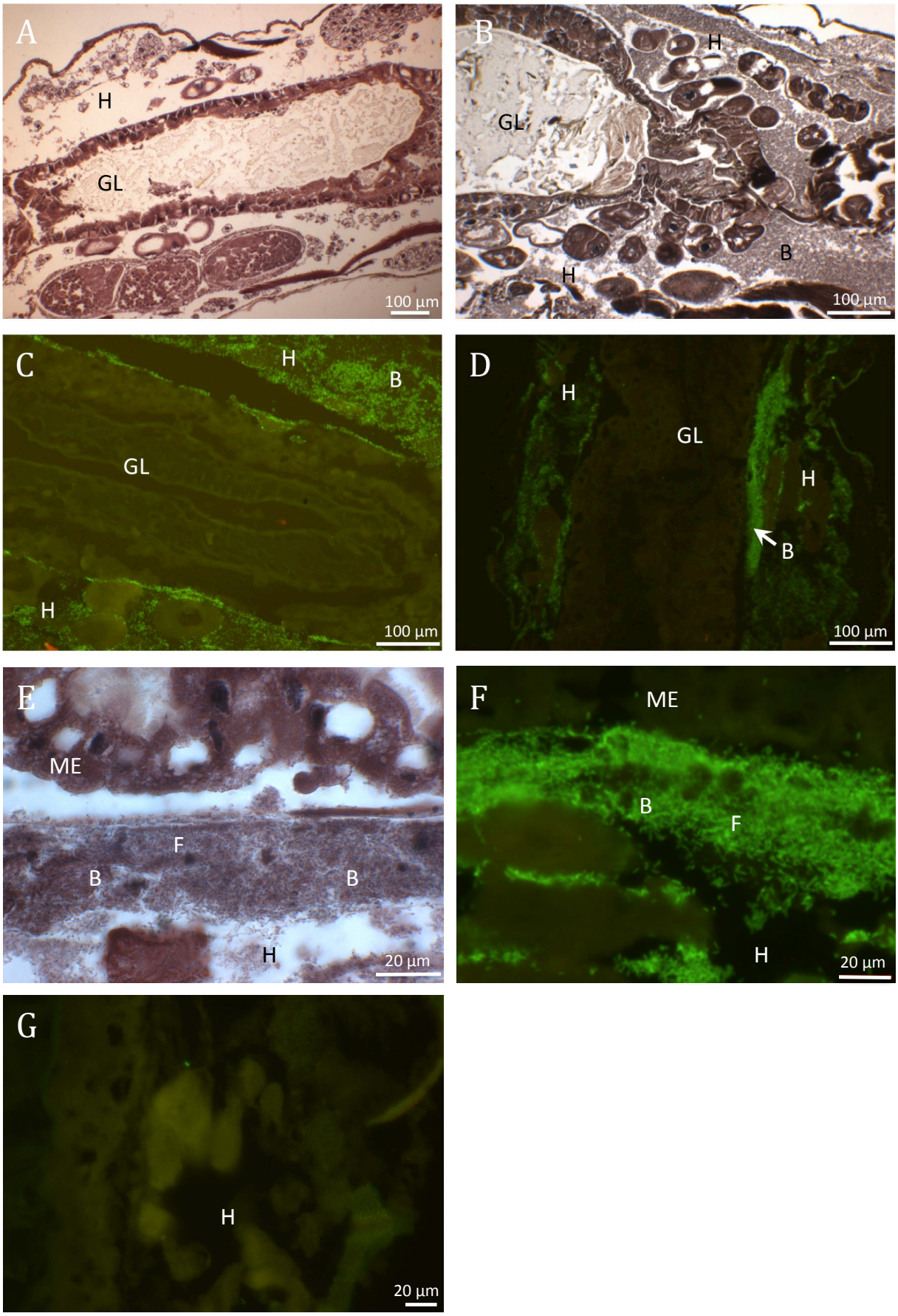


Figure 4 ***Pseudomonas protegens*** **CHA0 was rarely found in the gut of** ***Plutella xylostella*** **larvae.** One-week-old larvae were exposed to artificial diet inoculated with 10 µl of bacterial suspension of OD_{600} = 10. Larvae were fixed in Duboscq-Brazil's alcoholic Bouin's, embedded in histosec and cut into longitudinal sections of 6 µm. In most examined larvae, *P. protegens* CHA0-*gfp2* was not detected in the gut, but two exceptions are depicted in **(A)** and **(B-D)**. **(A)** Larva infected with *P. protegens* CHA0 1 dpi, bacteria are observed at the microvilli brush border. **(B-D)** *P. xylostella* larva infected with *P. protegens* CHA1176, a GFP expressing variant of CHA0, at 4 dpi. Bacteria can be seen in the lumen of the insect midgut **(B-D)**, but not in the hemolymph **(C)**. **(A, B)** Histological section stained with Heidenhain's iron hematoxylin. **(C, D)** Fixed tissue sections were stained with anti-GFP (green), because tissue fixation destroys intrinsic GFP fluorescence. **(B)** and **(D)** depict the same area in two consecutive sections of the same larva stained either with Duboscq-Brazil's alcoholic Bouin's or anti-GFP. **(D)** is a magnification of **(C)**. B, bacteria; GL, gut lumen; H, hemocoel; ME, midgut epithelium; MV, microvilli; PM, peritrophic matrix

In a few larvae, bacteria could be observed in the lumen of the insect midgut (Figure 4A-D), while the hemocoel was still free from the bacteria (Figure 4C). However, observing *P. protegens* CHA1176 in the gut was rather the exception than the rule. In most larvae harvested at early time-points (1-2 dpi) after infection bacteria were neither detected in the gut nor in the hemocoel. Moreover, insect organs of infected larvae did not differ from that of control larvae. At later time-points (3-4 dpi), however, several larvae were found to harbor large amounts of *P. protegens* CHA1176 in the hemocoel (Figure 5B-F). The Heidenhain's iron hematoxylin stains bacteria dark. Accordingly, the area where the hemolymph is supposed to be (transparent in the control larva, Figure 5A) is full of dark spots in the infected larva (Figure 5B). While the Haidenhain's iron hematoxylin stains bacteria nonspecifically, the use of anti-GFP antibodies revealed that the large amounts of bacteria observed in the hemolymph of several larvae are in fact *P. protegens* CHA1176 (Figure 5C, D and F). Figure 4B and D, Figure 5E and F and Figure 6B and D depict each two consecutive sections of the same larva, one stained with Heidenhain's iron hematoxylin and one stained with anti-GFP antibodies. Remarkably, the guts of larvae that had high amounts of *P. protegens* CHA0 or CHA1176 present in the hemocoel were free from bacteria (Figure 5B-D). Moreover, no extensive damage to the midgut epithelium was observed. In the hemocoel the bacteria were yet distributed all over, colonizing also the fat body cells (Figure 5E, F). Dense clusters of bacteria could be detected close to the basal lamina (Figure 5E, F). While in most larval samples from early time-points, but also in several harvested at late time-points, no *P. protegens* CHA1176 could be detected, moribund larvae were always packed with *P. protegens* CHA1176. These larvae were processed for microscopic analysis shortly before death would have occurred. Organs in moribund larvae were not distinctively recognizable anymore and *P. protegens* CHA1176 could be found all over the hemocoel, the fat body and the gut (Figure 6A-D).


A
H
GL
100 µm
B
H
GL
B
H
100 µm
C
H
B
GL
H
100 µm
D
H
GL
H
B
100 µm
E
ME
F
B
B
H
20 µm
F
ME
B
F
H
20 µm
G
H
20 µm

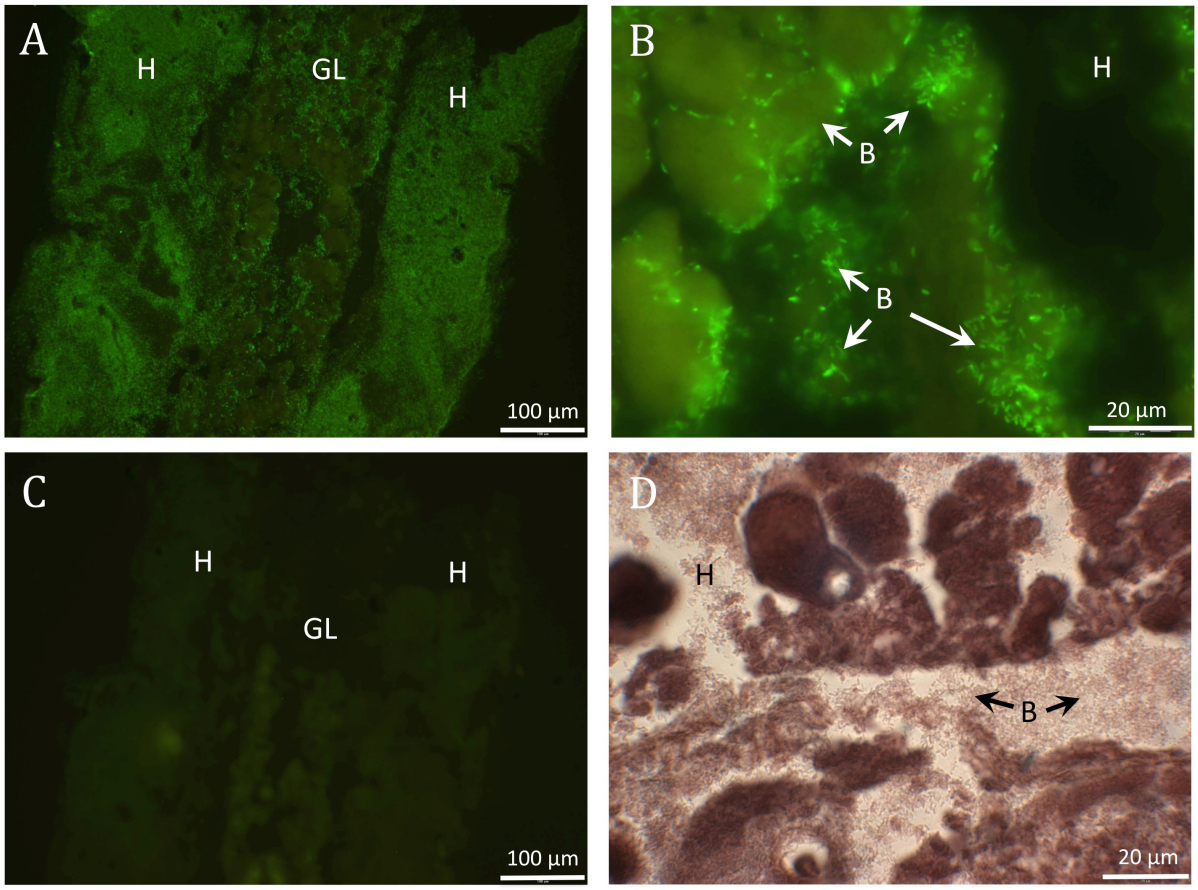


Figure 6 Moribund *Plutella xylostella* larvae are completely colonized by *Pseudomonas protegens* CHA1176, a GFP expressing variant of CHA0. One-week-old larvae were exposed to artificial diet inoculated with 10 µl of bacterial suspension of OD_{600} = 10. Pictures of one representative moribund larva (3 dpi) are shown. Larvae were fixed in Duboscq-Brazil's alcoholic Bouin's at 3dpi, embedded in histosec and cut into longitudinal sections of 6 µm. Consecutive sections were stained either with anti-GFP (green) **(A, B)**, without adding anti-GFP antibody **(C)** or with Heidenhain's iron hematoxylin **(D)**. **(B)** is a magnification of **(A)**. The use of anti-GFP antibodies was necessary, because fixation destroys intrinsic GFP fluorescence. B, bacteria; GL, gut lumen; H, hemocoel

Left side: Figure 5 *Pseudomonas protegens* CHA0 in the hemolymph of *Plutella xylostella larvae*. One-week-old larvae were exposed to artificial diet inoculated with 10 µl of bacterial suspension of OD_{600} = 10. Larvae were fixed in Duboscq-Brazil's alcoholic Bouin's, embedded in histosec and cut into longitudinal sections of 6 µm. **(A)** Control larva fed on bacteria-free diet, at 4 dpi. **(B)** *P. protegens* CHA0 infected larva at 3 dpi. **(C-G)** *P. xylostella* larvae infected with *P. protegens* CHA1176, a GFP expressing variant of CHA0, at 4 dpi **(C)** and at 3 dpi **(D-G)**. **(B-F)** Bacteria are present in the hemolymph and in fat body cells, but not in the gut. **(A, B, E)** Histological section stained with Heidenhain's iron hematoxylin. **(C, D, F)** Fixed tissue sections were stained with anti-GFP antibody (green), because tissue fixation destroys intrinsic GFP fluorescence. **(E)** and **(F)** depict the same area in two consecutive sections of the same larva stained either with Duboscq-Brazil's alcoholic Bouin's or anti-GFP. **(F)** is a magnification of **(D)**. **(G)** Control in which no anti-GFP antibody was added. B, bacteria; F, fat body; GL, gut lumen; H, hemocoel; ME, midgut epithelium

DISCUSSION

This study investigated for the first time the biocontrol potential of *P. protegens* CHA0 against a root feeding insect pest and examined whether CHA0 can use the insect host as a vector for dispersal, for which bacterial persistence in the insect would be a prerequisite. Our results provide evidence for the occurrence of transstadial transmission of *P. protegens* CHA0 and for potential insect-mediated dispersal by adult *D. radicum* emerging from larvae feeding on CHA0 colonized roots. No effect of CHA0 on survival of larvae and pupae was detected, but the fact that several adults emerging from the CHA0 treatment exhibited morphological defects still suggests a negative impact of CHA0 on *D. radicum*.

Biocontrol activity against *D. radicum*

If *P. protegens* CHA0, as observed under the tested conditions, does not affect survival of *D. radicum* larvae, which is the crop damage causing developmental stage, no immediate control of a *D. radicum* infestation might be achieved. However, CHA0-induced morphological defects in adults could reduce *D. radicum* reproduction due to the inability of some adults to fly. This would result in a decline of pest populations and finally reduce damage caused by the following generations. Moreover, *P. protegens* CHA0 might be of relevance as an opportunistic pathogen, infecting weakened individuals.

Morphological defects in adult flies were also observed upon infections of *D. melanogaster* larvae by the close relative of CHA0, *P. protegens* Pf-5 (Olcott *et al.*, 2010; Loper *et al.*, 2016). Individuals that survived an infection by Pf-5 as larva and developed until the adult stage showed mainly eye defects, but also abnormalities of the wings. The eye defects caused by Pf-5 are correlated to the production of rhizoxin (Loper *et al.*, 2016). *P. protegens* CHA0 does not produce rhizoxin, which might explain the absence of eye defects in our experiments. However, the wing defects caused by CHA0 seem to be more dramatic than those reported for Pf-5 (Olcott *et al.*, 2010), which might be attributed to the different insect species or to strain specific differences among pseudomonads. The hypothesis of a strain specific effect is supported by pilot experiments with CHA0-fed *P. brassicae* larvae. In this insect species, the *P. protegens* CHA0 infection was also causing strong wing defects, but no eye defects in emerging

adults (C. Tinguely, personal communication). Therefore, future more in-depth studies on morphological defects, especially wing deformations, will be of great interest for a better understanding of insecticidal activity of *P. protegens* CHA0.

The observation that in the cauliflower pot experiments *D. radicum* survival was not affected by the presence of *P. protegens* CHA0 on the roots is in contrast to former studies, reporting high mortality rates in several insect species upon ingestion of *P. protegens* CHA0 (Ruffner *et al.*, 2013; Flury *et al.*, 2016). One explanation is that *D. radicum* is much less susceptible to the bacterium than the tested lepidopteran species. However, in the oral feeding assays with Lepidoptera, larvae were kept in small cages and were 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 the cauliflower roots were presumably very low, since larvae burrow into the root and *P. protegens* CHA0 colonizes mainly the root surface (Troxler *et al.*, 1997). Infections of *D. melanogaster*, which belongs to the same insect order as *D. radicum*, by *P. protegens* Pf-5 were found to be dose-dependent (Olcott *et al.*, 2010). Since the presumably low amounts of *P. protegens* CHA0 taken up by *D. radicum* in our system already caused morphological defects in part of the flies, we hypothesize that mortality rates of larvae and pupae could be increased by higher amounts of bacteria or by exposing *D. radicum* to an additional stress factor. If *P. protegens* CHA0 is directly injected into the hemocoel, it can rapidly multiply and kill the insect (Péchy-Tarr *et al.*, 2008). Therefore, combining the bacteria with an organism that could facilitate the access to the hemocoel, such as entomopathogenic fungi or nematodes may result in good biocontrol effects. Finally, other root pests than *D. radicum* might be more susceptible to *P. protegens* CHA0, since related strains seem to exhibit host specificity (Rangel *et al.*, 2016). The oral toxin produced by a *Pseudomonas chlororaphis* strain, for instance, was found to kill corn rootworms, but had no effect against several other insect species (Schellenberger *et al.*, 2016).

Insect colonization and transstadial persistence

The presented findings suggest that transstadial transmission of *P. protegens* CHA0 in insects is a common phenomenon, but to which extent the bacteria are transmitted is dependent on the insect species. Transstadial transmission (from larva to adult) of bacteria that do not exhibit an intracellular lifestyle, which is common for endosymbionts (Kikuchi, 2009; Engel and Moran, 2013), is reported for several insect species (Radvan, 1960; Greenberg and Klowden, 1972; Moll *et al.*, 2001; Chavshin *et al.*, 2015). However, in other cases bacteria were lost during pupal stage, before adult emergence (Leach, 1934; Radvan, 1960; Greenberg and Klowden, 1972; Moll *et al.*, 2001).

Based on our results we hypothesize that the oral uptake of *P. protegens* CHA0 by insect larvae can result in either of three main scenarios: (1) Ingested *P. protegens* CHA0 is able to colonize the larval digestive system to a certain extent without affecting larval fitness, but becomes eradicated before or at onset of metamorphosis and is hence not detectable in pupae and adults, (2) *P. protegens* CHA0 does not affect larval survival, but is able to persist throughout metamorphosis until the pupal and the adult stage and occasionally causes defects in adults, and (3) *P. protegens* CHA0 can successfully infect the larvae, reaches the hemocoel and multiplies to very high numbers, which leads to larval or at latest pupal death.

Larvae falling into scenario one seem to be resistant to infection by *P. protegens* CHA0 as they are able to eradicate the bacteria during the larval stage. This seems to be the case for many *P. brassicae* larvae (Figure 3C), since pupae were found to be free from *P. protegens* CHA0 apart from a few exceptions of dead pupae carrying the bacteria. Although most *P. brassicae* larvae seem to eliminate *P. protegens* CHA0 before the pupal stage, some emerging adults exhibited morphological defects (C. Tinguely, personal communication), indicating negative effects of *P. protegens* CHA0 on *P. brassicae* development. We have to add, that only *P. brassicae* larvae fed at the fourth instar seem to remain mostly unaffected by CHA0. Larvae fed at earlier instars are highly susceptible to the bacterium, since up to 70% larval mortality was obtained in feeding assays with second and third instars (Kupferschmied *et al.*, 2013)(P. Vesga and M. Péchy-Tarr, personal communication).

In scenario two, *P. protegens* CHA0 is transstadially transmitted, without affecting insect survival as we observed for *D. radicum* and *O. sulcatus*. Surface

sterilization of insects prior to extraction revealed that the bacteria are inside larvae, pupae and in some cases even adults, but about their exact location we can only speculate. At the larval stage the bacteria presumably colonize the gut, but do not invade the hemocoel. Due to repeated molting, 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 (Engel and Moran, 2013). To survive until the adult stage *P. protegens* CHA0 somehow has to evade the dramatic restructuring during metamorphosis. In dipteran species for instance, Malpighian tubules were hypothesized to provide a hiding place for gut bacteria, since they remain intact during metamorphosis (Chavshin *et al.*, 2013; Chavshin *et al.*, 2015). Indeed, *Pseudomonas* strain BND-YL1 was found to colonize the Malpighian tubules of its insect host *Anopheles stephensi* during larval, pupal and adult stage (Chavshin *et al.*, 2015). In an early histological study, bacterial persistence in pupae of *Delia platura*, a close relative of *D. radicum,* was studied during development (Leach, 1934). Bacteria were observed in the intestine and in the space between the prepupal cuticle and the true pupa. During histolysis bacteria could not be detected for a limited period, but they reappeared before imago emergence (Leach, 1934). Persistence of *P. protegens* CHA0 in insects of different orders such as *P. xylostella* (Lepidoptera), *D. radicum* (Diptera) and *O. sulcatus* (Coleoptera), each of which harbors specific anatomical and developmental features, might rely on different strategies, which remain to be investigated. To unravel the fate of CHA0 during metamorphosis, detailed microscopic investigations would be needed.

Finally, in the last scenario, larvae are highly susceptible to *P. protegens* CHA0 as observed in this and earlier studies for *P. xylostella* (Ruffner *et al.*, 2013; Flury *et al.*, 2016) and in earlier studies also for *S. littoralis* and *Heliothis virescens* larvae (Ruffner *et al.*, 2013). In highly susceptible larvae, *P. protegens* CHA0 multiplies to very high numbers as shown in Figure 3 and reported earlier (Péchy-Tarr *et al.*, 2008; Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2013; Flury *et al.*, 2016) leading to larval death. Extraction of entire larvae, however, does not allow any conclusion on the localization of the bacteria. The here presented microscopy method enabled the visualization of *P. protegens* CHA1176 during the insect infection and, in parallel, the observation of histopathological changes in *P. xylostella* larvae. First 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 (Figure 4, Figure 5).

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 are able to multiply exponentially causing a fatal septicemia. The use of the insect body as a mass replication vessel is supported by the pictures of moribund larvae in Figure 6 as well as by the very high bacterial counts in dead individuals of *P. xylostella* and *P. brassicae* extracted in Figure 3B and C. The pictures presented here only give 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 more in-depth microscopy studies.

Insect-mediated dispersal of *P. protegens* CHA0

The here presented experiments give first evidence that *P. protegens* CHA0 can be dispersed by *D. radicum* to a new host plant. A summary of a potential transmission cycle is depicted in Figure 2. *P. protegens* CHA0, which colonizes plant roots, becomes internalized by root feeding *D. radicum* larvae. It persists inside the larvae until the pupal stage and eclosing flies are able to transmit *P. protegens* CHA0 to the roots of a new host plant (Figure 2). This could, for instance, occur during egg deposition by female flies. Since eggs are laid on the ground next to the plant stem, the bacteria would directly encounter a new soil habitat. Alternatively, *P. protegens* CHA0 might be transmitted to plant shoots and get washed into the soil by rainfall. In the following, *P. protegens* CHA0 colonizes the roots of the new host plant from where it can again infect larvae that hatch from deposited eggs (Figure 2).

Transmission by healthy looking flies was observed, although *P. protegens* CHA0-*gfp2* was only detected in flies with morphological defects. An explanation for this observation could be that flies carry only very few bacteria, which were not detectable by our method having a detection limit between 10 and 100 bacterial cells per insect. The transmission of a single bacterial cell of *P. protegens* CHA0-*gfp2*, however, could be enough to result in colonization of plant roots in the transmission microcosms, since plants were grown axenically, and the introduced bacteria would not encounter competition with other microbes. Bacterial transmission despite low infestation of the insect was also observed by Snyder *et al.* (1998), who found that individual *D. undecimpunctata* subsp. *howardi* adults carrying only 10 cfu of *P. chlororaphis* L11 were transmitting the bacteria to a new host plant in 50% of the cases. In our

experiments the transfer was observed in 90% of the cases, which indicates that most adult flies carry the bacterium, although we could not detect it by our extraction method. Another explanation for the failure to detect CHA0 in adult flies could be that the bacteria were present inside the fly body at eclosion but were lost soon after. Midgut bacteria might become enclosed in the meconium, the sloughed, degenerating larval midgut enveloped by a peritrophic matrix, which becomes excreted by the adult insect as the first feces soon after eclosion (Moll *et al.*, 2001; Broderick and Lemaitre, 2012). Alternatively, flies might become contaminated by *P. protegens* CHA0 cells that are associated to the pupal case during hatching. In such a scenario, flies would carry the bacteria on their surface, a dry environment with high exposure to UV radiation, which is hostile to long-term survival of *P. protegens* CHA0. However, in both cases, CHA0 on the surface or in the meconium, the bacteria might have been carried long enough for transmission, but too short for detection, since extraction of flies was conducted about one to three days after eclosion. In summary, transmission of *P. protegens* CHA0-*gfp2* to a new host strongly indicates that flies are carrying the bacteria, but the exact way of transmission is still speculative.

The fact that dispersal of *P. protegens* CHA0 from the roots of one plant to the roots of another plant by an insect seems to be possible indicates that insects may serve *P. chlororaphis* subgroup bacteria not only as hosts, but also as vectors. As such they 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 (Nadarasah and Stavrinides, 2011); 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 (Chatterjee *et al.*, 2008). Still, in some associations the plant-pathogen is also insect-pathogenic. *P. syringae* B728a is able to cause high mortality rates in the pea aphid and at the same time, bacteria are found to be excreted with honeydew and can thereby be dispersed by moving aphids (Stavrinides *et al.*, 2009). Since *P. protegens* CHA0 was found to persist in pupae, the hibernating stage of *D. radicum* and *O. sulcatus*, it might use this alternative host also as a habitat for overwintering and protection from cold temperatures, as it was described for a *Pseudomonas fluorescens* strain in the Colorado potato beetle (Castrillo *et al.*,

2000). The discovery of persistence of *P. protegens* CHA0 in insects throughout different developmental stages and its dispersal to a new host plant adds new and intriguing aspects to the ecology of fluorescent pseudomonads with insecticidal activity. It will be highly interesting to unravel in future studies how these bacteria adapt to and make use of this alternative host and how they interact with different insect species.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHORS AND CONTRIBUTORS

PF, RK, CK and MM conceived and designed the research. RK, CK and MM supervised the study. PF together with AD, DB and AT performed the *D. radicum* experiments. PF and DB performed the *P. xylostella* experiments. PV, CT and JS performed the *P. brassicae* experiments. PF, RK and CU conducted the microscopy experiments. PF analyzed the data.

FUNDING

This study was financed by grants obtained from the Swiss National Foundation for Scientific Research SNSF (Projects 31003A-138248, 31003A-159520). The research stay of PF at JKI Darmstadt was supported by a grant obtained from Walter Hochstrasser-Stiftung, Zürich, Switzerland.

ACKNOWLEDGEMENTS

We gratefully acknowledge Matthias Lutz, Jaka Razinger, Anne-Marie Cortesero, Syngenta Crop Protection and the Environmental Systems Sciences group of ETH Zurich for providing insect material. Moreover, we 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 Astrid Keller for her 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 Sandra Siegfried for technical assistance.

REFERENCES

Benjamin J. L., Sumpter R., Jr., Levine B., Hooper L. V. (2013). Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell host & microbe* 13**:** 723-734.

Berg G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 84**:** 11-18.

Broderick N. A., Lemaitre B. (2012). Gut-associated microbes of *Drosophila melanogaster*. *Gut microbes* 3**:** 307-321.

Castrillo L. A., Lee R. E., Jr., Lee M. R., Rutherford S. T. (2000). Identification of ice-nucleating active *Pseudomonas fluorescens* strains for biological control of overwintering Colorado potato beetles (Coleoptera: Chrysomelidae). *J Econ Entomol* 93**:** 226-233.

Chatterjee S., Almeida R. P. P., Lindow S. (2008). Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. *Annu Rev Phytopathol* 46**:** 243-271.

Chavshin A. R., Oshaghi M. A., Vatandoost H., Yakhchali B., Raeisi A., Zarenejad F. (2013). *Escherichia coli* expressing a green fluorescent protein (GFP) in *Anopheles stephensi*: a preliminary model for paratransgenesis. *Symbiosis* 60**:** 17-24.

Chavshin A. R., Oshaghi M. A., Vatandoost H., Yakhchali B., Zarenejad F., Terenius O. (2015). Malpighian tubules are important determinants of *Pseudomonas* transstadial transmission and longtime persistence in *Anopheles stephensi*. *Parasites & vectors* 8**:** 36.

David W. A. L., Gardiner B. O. (1965). Rearing *Pieris brassicae* L Larvae on a semi-synthetic diet. *Nature* 207**:** 882-883.

Devi K. K., Kothamasi D. (2009). *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting cytochromecoxidase of the termite respiratory chain. *FEMS Microbiol Lett* 300**:** 195-200.

Engel P., Moran N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev* 37**:** 699-735.

Flury P., Aellen N., Ruffner B., Pechy-Tarr M., Fataar S., Metla Z. *et al.* (2016). Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics. *The ISME journal* 10**:** 2527-2542.

Greenberg B., Klowden M. (1972). Enteric bacterial interactions in insects. *Am J Clin Nutr* 25**:** 1459-1466.

Holt R. D., Hochberg M. E. (1997). When is biological control evolutionarily stable (or is it)? *Ecology* 78**:** 1673-1683.

Iavicoli A., Boutet E., Buchala A., Metraux J. P. (2003). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Mol Plant-Microbe Interact* 16**:** 851-858.

Jang J. Y., Yang S. Y., Kim Y. C., Lee C. W., Park M. S., Kim J. C. *et al.* (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J Agric Food Chem* 61**:** 6786-6791.

Keel C., Voisard C., Berling C. H., Kahr G., Défago G. (1989). Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* 79**:** 584-589.

Keel C., Schnider U., Maurhofer M., Voisard C., Laville J., Burger U. *et al.* (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0 - Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant-Microbe Interact* 5**:** 4-13.

Keel C. (2016). A look into the toolbox of multi-talents: insect pathogenicity determinants of plant-beneficial pseudomonads. *Environ Microbiol* 18**:** 3207-3209.

Kikuchi Y. (2009). Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes Environ* 24**:** 195-204.

King E. O., Ward M. K., Raney D. E. (1954). 2 simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* 44**:** 301-307.

Kupferschmied P., Maurhofer M., Keel C. (2013). Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Frontiers in plant science* 4**:** 287.

Kupferschmied P., Péchy-Tarr M., Imperiali N., Maurhofer M., Keel C. (2014). Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. *PLoS Path* 10**:** e1003964.

Kupferschmied P., Chai T., Flury P., Blom J., Smits T. H., Maurhofer M. *et al.* (2016). Specific surface glycan decorations enable antimicrobial peptide resistance in plant-beneficial pseudomonads with insect-pathogenic properties. *Environ Microbiol* [Epub ahead of print].

Lamb T. G., Tonkyn D. W., Kluepfel D. A. (1996). Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. *Canadian Journal of Microbiology* 42**:** 1112-1120.

Leach J. G. (1934). The method of survival of bacteria in the puparia of the seedcorn maggot (*Hylemyia cilicrura* Rond.). *J Appl Entomol* 20**:** 150-161.

Lemanceau P., Maurhofer M., Défago G. (2007). Contribution of studies on suppressive soils to the identification of bacterial biocontrol agents and to the knowledge of their modes of action. In: Gnanamanickam S. (ed). *Plant-associated bacteria*. Springer: Dordrecht, The Netherlands. pp 231-267.

Loper J. E., Henkels M. D., Rangel L. I., Olcott M. H., Walker F. L., Bond K. L. *et al.* (2016). Rhizoxin, orfamide A, and chitinase production contribute to the toxicity of *Pseudomonas protegens* strain Pf-5 to *Drosophila melanogaster*. *Environ Microbiol* 18**:** 3509-3521.

Maurhofer M., Hase C., Meuwly P., Metraux J. P., Defago G. (1994a). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0 - Influence of the *GacA* gene and of pyoverdine production. *Phytopathology* 84**:** 139-146.

Maurhofer M., Keel C., Haas D., Defago G. (1994b). Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber. *Eur J Plant Pathol* 100**:** 221-232.

Maurhofer M., Keel C., Haas D., Defago G. (1995). Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHA0 with enhanced antibiotic production. *Plant Pathol* 44**:** 40-50.

Maurhofer M., Reimmann C., Schmidli-Sacherer P., Heeb S., Haas D., Défago G. (1998). Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. *Phytopathology* 88**:** 678-684.

Moll R. M., Romoser W. S., Modrzakowski M. C., Moncayo A. C., Lerdthusnee K. (2001). Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *J Med Entomol* 38**:** 29-32.

Mulet M., Gomila M., Scotta C., Sanchez D., Lalucat J., Garcia-Valdes E. (2012). Concordance between whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry and multilocus sequence analysis approaches in species discrimination within the genus *Pseudomonas*. *Syst Appl Microbiol* 35**:** 455-464.

Nadarasah G., Stavrinides J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol Rev* 35**:** 555-575.

Olcott M. H., Henkels M. D., Rosen K. L., Walker F. L., Sneh B., Loper J. E. *et al.* (2010). Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. *PloS one* 5**:** e12504.

Péchy-Tarr M., Bruck D. J., Maurhofer M., Fischer E., Vogne C., Henkels M. D. *et al.* (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ Microbiol* 10**:** 2368-2386.

Péchy-Tarr M., Borel N., Kupferschmied P., Turner V., Binggeli O., Radovanovic D. *et al.* (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ Microbiol* 15**:** 736-750.

Radvan R. (1960). Persistence of bacteria during development in flies. *Folia Microbiol* 5**:** 50-56.

Rangel L. I., Henkels M. D., Shaffer B. T., Walker F. L., Davis E. W., 2nd, Stockwell V. O. *et al.* (2016). Characterization of toxin complex gene clusters and insect toxicity of bacteria representing four subgroups of *Pseudomonas fluorescens*. *PloS one* 11**:** e0161120.

Razinger J., Lutz M., Schroers H.-J., Urek G., Grunder J. (2014). Evaluation of insect associated and plant growth promoting fungi in the control of cabbage root flies. *J Econ Entomol* 107**:** 1348-1354.

Ruffner B. (2013). Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis, ETH Zurich, Zurich, Switzerland.

Ruffner B., Péchy-Tarr M., Ryffel F., Hoegger P., Obrist C., Rindlisbacher A. *et al.* (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ Microbiol* 15**:** 751-763.

Ruffner B., Pechy-Tarr M., Hofte M., Bloemberg G., Grunder J., Keel C. *et al.* (2015). Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* 16**:** 609.

Schellenberger U., Oral J., Rosen B. A., Wei J.-Z., Zhu G., Xie W. *et al.* (2016). A selective insecticidal protein from *Pseudomonas* for controlling corn rootworms. *Science* [Epub ahead of print].

Snyder W. E., Tonkyn D. W., Kluepfel D. A. (1998). Insect-mediated dispersal of the rhizobacterium *Pseudomonas chlororaphis*. *Phytopathology* 88**:** 1248-1254.

Snyder W. E., Tonkyn D. W., Kluepfel D. A. (1999). Transmission of a genetically engineered rhizobacterium by grasshoppers in the laboratory and field. *Ecol Appl* 9**:** 245-253.

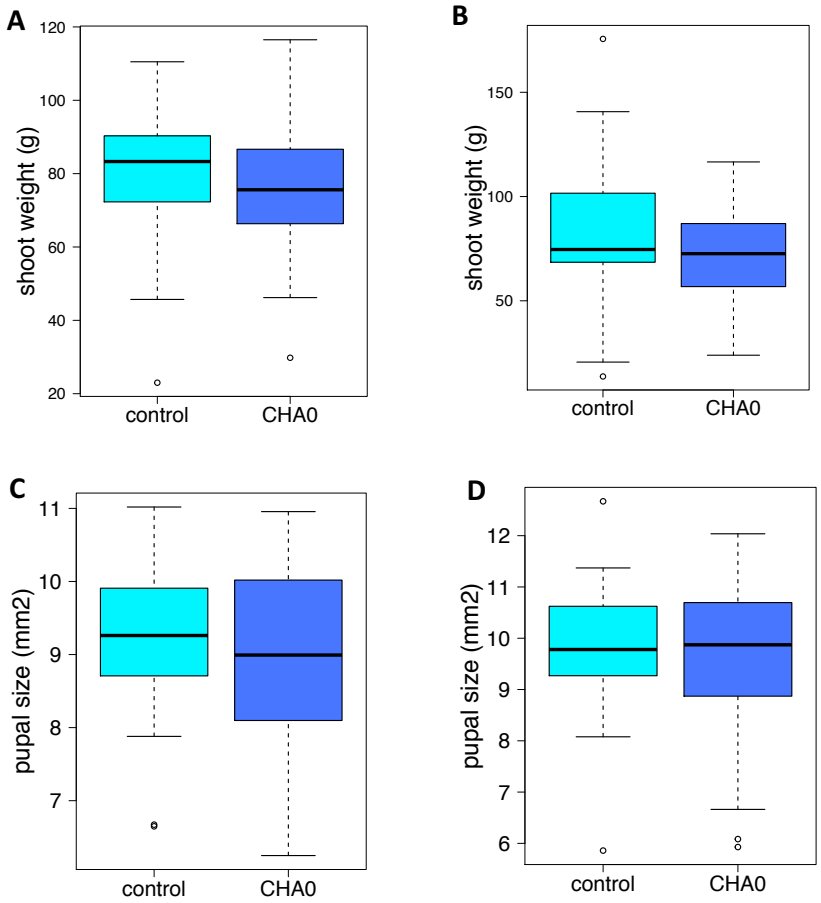
Stavrinides J., McCloskey J. K., Ochman H. (2009). Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. *Appl Environ Microbiol* 75**:** 2230-2235.

Stutz E. W., Défago G., Kern H. (1986). Naturally-occuring fluorescent pseudomonads involved in suppression of black root-rot of tobacco. *Phytopathology* 76**:** 181-185.

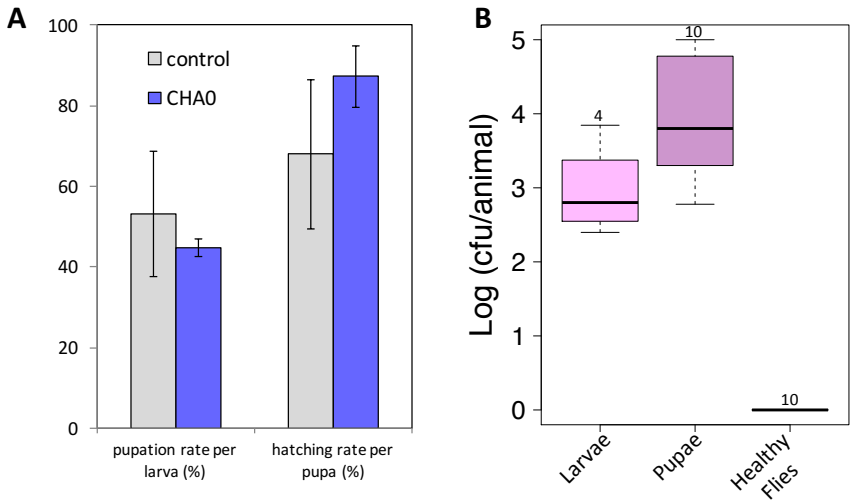
Troxler J., Berling C. H., MoenneLoccoz Y., Keel C., Defago G. (1997). Interactions between the biocontrol agent *Pseudomonas fluorescens* CHA0 and *Thielaviopsis basicola* in tobacco roots observed by immunofluorescence microscopy. *Plant Pathol* 46**:** 62-71.

Von Felten A., Défago G., Maurhofer M. (2010). Quantification of *Pseudomonas fluorescens* strains F113, CHA0 and Pf153 in the rhizosphere of maize by strain-specific real-time PCR unaffected by the variability of DNA extraction efficiency. *J Microbiol Methods* 81**:** 108-115.

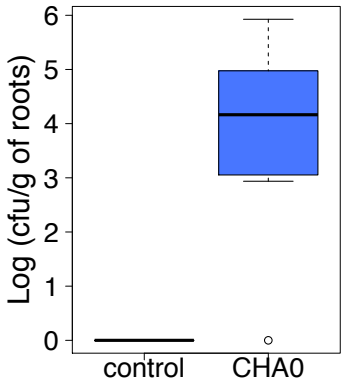
SUPPLEMENTARY INFORMATION



Supplementary Figure S1 ***Pseudomonas protegens*** **CHA0-*gfp2* does neither increase the shoot weight of cauliflower plants infested with *D. radicum* larvae nor reduces pupal size of the insect.** Roots of cauliflower plants were inoculated with a suspension of *P. protegens* CHA0-*gfp2* (CHA0) or 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 shoot weight of cauliflower plants **(A, B)** as well as size of emerged pupae **(C, D)** was assessed. For both parameters no significant difference between control and 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 A and B are depicted in **(A, C)** and **(B, D)**, respectively.



Supplementary Figure S2 *Pseudomonas protegens* CHA0-*gfp2* persists in larvae and pupae of *Delia radicum*, but does not affect insect survival. This experiment is a repetition of that depicted in Figure 1. The roots of cauliflower plants (four trays per treatment, each containing twelve pots) were either inoculated with *P. protegens* CHA0-*gfp2* (CHA0) or mock (control). Five freshly hatched *D. radicum* larvae were added per pot. After 3 weeks, emerging pupae as well as remaining larvae were collected. **(A)** Pupation rate and hatching rate of *D. radicum* did not significantly differ between control and *P. protegens* CHA0-*gfp2* treatment ($p < 0.05$; Student's t test). Error bars depict standard deviation of the mean of four replicate trays. **(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*. Insects were homogenized in 0.9% NaCl and bacterial numbers assessed by plating serial dilutions on selective medium. Larvae and pupae were surface sterilized prior to homogenization. Identity of growing colonies was verified by checking for GFP-expression using fluorescence microscopy. Numbers above boxes indicate sample size. No *P. protegens* CHA0-*gfp2* was detected in individuals emerging from the control treatment (data not shown).



Supplementary Figure S3 *Pseudomonas protegens* CHA0 taken up by root-feeding *Delia radicum* larvae can be dispersed to a new host plant. This experiment is a repetition of that depicted in Figure 1C. Root colonization of rapeseed plantlets in transfer systems. Pupae emerging from larvae that had fed on roots of cauliflower plants colonized by *P. protegens* CHA0-*gfp2* (CHA0) or free from these bacteria (control) were placed into closed sterile systems (3-4 pupae per system) containing rapeseed plantlets grown in a sand-vermiculite mix. To prevent transfer of bacteria by the pupae, they were kept separately from plants and substrate. Emerging flies flew around inside the systems and in some cases also laid eggs. Nine days after first flies had started to hatch, root systems were collected and bacterial colonization levels were assessed by dilution plating. In the CHA0 treatment roots in eight out of nine systems became colonized with *P. protegens* CHA0-*gfp2*. Identities of growing colonies were verified by checking for GFP expression using fluorescence microscopy. Numbers above boxes indicate sample size.

Supplementary Table S1 Vitamin stock solution for *Pieris brassicae* diet

Nicotinic acid	600 mg
Calcium pantothenate	600 mg
Riboflavin (B2)	300 mg
Thiamine hydrochloride (B1)	150 mg
Pyridoxine hydrochloride (B6)	150 mg
Folic acid	150 mg
D-Biotin	12 mg
Cyanocobalamine (B12)	1.2 mg
H_2O	100 ml

Supplementary Table S2 Salt stock solution for *Pieris brassicae* diet

$CaCO_3$	120 g
K_2HPO_4	129 g
$Ca(H_2PO_4)_2 \cdot H_2O$	30 g
$MgSO_4 \cdot 7H_2O$	40.8 g
NaCl	67 g
$FeH_5C_6O_7 \cdot H_2O$	8.15 g
KI	0.32 g
$MnSO_4 \cdot H_2O$	2 g
$ZnCl_2$	0.10 g
$CuSO_4 \cdot 5H_2O$	0.12 g
H_2O	1000 ml

CHAPTER 5

General Discussion

5 GENERAL DISCUSSION

5.1. The *Pseudomonas fluorescens* group – a heterogeneous cluster of strains with various interesting features

In the presented thesis, we investigated the characteristics of *Pseudomonas fluorescens* group bacteria, with a strong focus on insecticidal activity. The *P. fluorescens* group comprises a vast diversity of heterogeneous strains exhibiting several interesting features, a summary of which is illustrated in Figure 1. Many strains are reported to exhibit plant beneficial traits – disease suppression, growth promotion and/or induction of systemic resistance (Haas and Défago, 2005; De Vleesschauwer and Höfte, 2009) – and certain strains were further discovered to have insecticidal activity (Péchy-Tarr *et al.*, 2008; Olcott *et al.*, 2010; Ruffner *et al.*, 2013; Ruffner *et al.*, 2015; Rangel *et al.*, 2016), (Chapter 2). Most research groups study one or a few strains under their specific experimental conditions. This circumstance makes it difficult to compare the properties of different strains and to draw conclusions on their distribution within the *P. fluorescens* group. In Chapter 2 of this thesis, we sequenced the genomes of 24 strains phylogenetically spread over the entire *P. fluorescens* group and characterized them experimentally for their plant beneficial and their insecticidal traits. This allowed us to obtain a more complete picture on the distribution of these traits throughout the *P. fluorescens* group, and the genomic data further provided us with a valuable tool for investigations on their genetic basis. The ability to suppress root pathogens, in vitro and in vivo, was found to be spread all over the *P. fluorescens* group, though it was not exhibited by all strains. Similarly, phosphate solubilization, which contributes to plant growth promotion, was assessed in vitro and was found in several, but not all, strains, phylogenetically clustering in different subgroups (data not shown). Moreover, preliminary data on the ability to induce resistance against above-ground pathogens and insect pests has been generated by several bachelor and master theses projects and is under further investigation (T. Löser, personal communication). Finally, all strains were tested for injectable and oral insecticidal activity, which revealed that strong insecticidal activity is unique to the *Pseudomonas chlororaphis* subgroup, also called sub-clade 1. However, moderate insecticidal activity was also found in strains of the *Pseudomonas fluorescens* subgroup/ sub-clade 3. After gaining this broad overview on features of the *P. fluorescens* group, we then concentrated on investigating insecticidal activity of the

P. chlororaphis subgroup in more detail in order to understand the underlying mechanisms, to unravel unknown facets of their insect-associated lifestyle and to explore their biocontrol potential against insect pests, the main findings of which are discussed in the following Chapters.

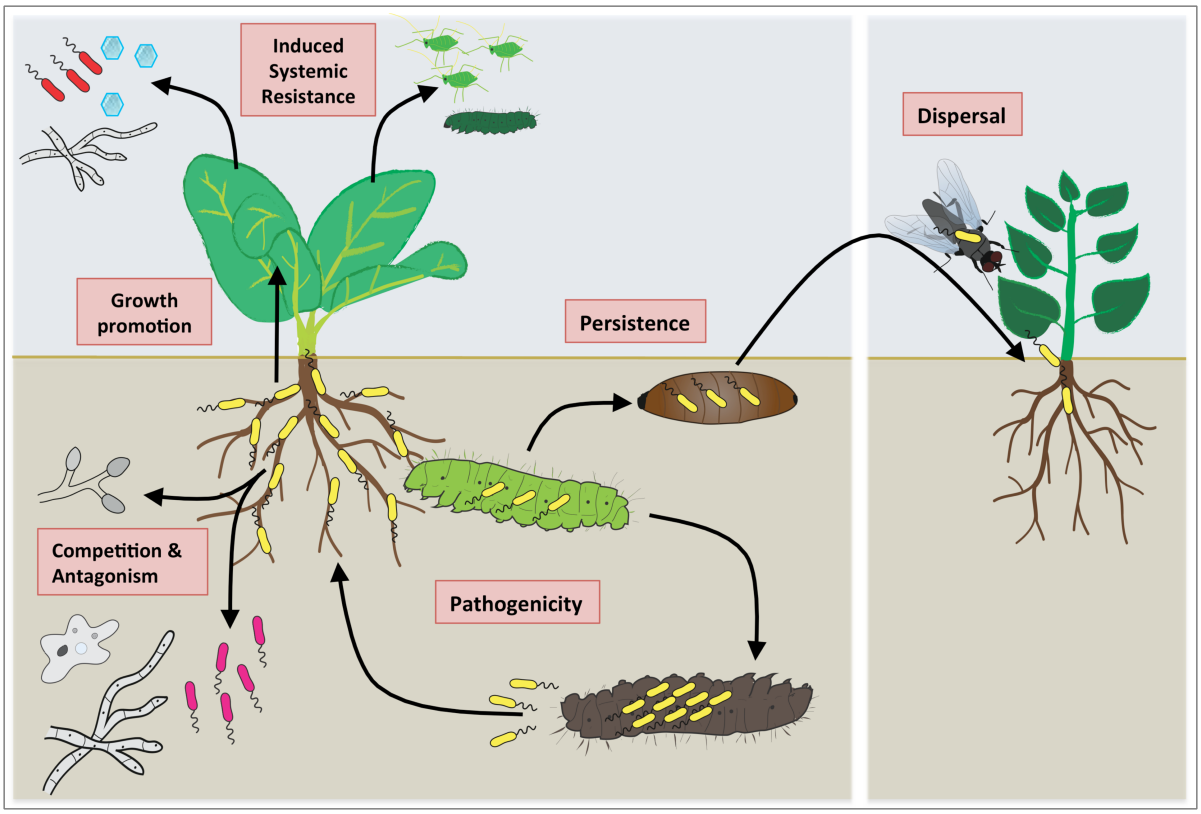


Figure 1 Plant-beneficial and insecticidal features exhibited by *Pseudomonas fluorescens* group bacteria. Root-colonizing fluorescent pseudomonads are reported to suppress root diseases by direct competition for nutrients and space and by antagonizing pathogenic microorganisms through the production of antimicrobial metabolites. Additional plant-beneficial traits are the promotion of plant growth and the induction of defense responses rendering the entire plant more resistant to pathogen and insect attacks. A distinct group of strains further exhibits insect pathogenicity. Upon ingestion by larvae, they are able to cause a lethal infection, during which they multiply to very high numbers. Finally, for strain *Pseudomonas protegens* CHA0 it was further demonstrated that it is able to persist inside the insect throughout different lifestages and can use this alternative host as a vector for dispersal.

5.2. Mechanisms of insect colonization and killing

When this thesis was started, Fit was the only factor for which a contribution to insecticidal activity of *P. chlororaphis* subgroup bacteria had been clearly demonstrated (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013). Since then our understanding of insect pathogenicity of fluorescent pseudomonads substantially increased and this thesis along with other studies revealed several additional contributing factors (Keel, 2016; Kupferschmied *et al.*, 2016; Loper *et al.*, 2016; Schellenberger *et al.*, 2016)(Chapter 2, Chapter 3), which supports the current view that insecticidal activity of fluorescent pseudomonads is multifactorial. A summary of the factors and the two regulatory systems with a demonstrated role in insecticidal activity of the *P. chlororaphis* subgroup is presented in Table 1. The different pathogenicity factors presumably come into action at different time points during the infection process. Accordingly, some were found to be crucial for full virulence in oral infections but not when bacteria are directly injected into the hemocoel, which suggests a role in the insect gut, while others seem to exhibit toxic effects in the hemolymph or to contribute to evasion of the insect immune system.

Upon ingestion by an insect larva, *P. chlororaphis* subgroup bacteria survive in the intestine, but exponential growth seems to occur only once they infested the hemocoel (Chapter 4). In order to get there, they have to breach the gut barrier. In other entomopathogenic bacteria this includes the production of chitinases, phospholipases and proteases (Jurat-Fuentes and Jackson, 2012). Considering the reported effects of these enzymes (Lysenko, 1972; Ikezawa *et al.*, 1989; Regev *et al.*, 1996; Sampson and Gooday, 1998; Jurat-Fuentes and Jackson, 2012; ffrench-Constant and Dowling, 2014), chitinase C produced by *Pseudomonas protegens* may initially add to the degradation of the peritrophic matrix followed by the action of another exoenzyme, phospholipase C, which possibly attacks the midgut epithelial cells. While chitinase and phospholipase deficient mutants exhibited decreased insect pathogenicity (Loper *et al.*, 2016)(Chapter 2)(Table 1), no such effect was observed for the two proteases AprA and AprX (Loper *et al.*, 2016)(Chapter 2, Chapter 3). In contrast, different classes of cyclic lipopeptides contribute to insect pathogenicity (Loper *et al.*, 2016)(Chapter 3). Whether they do so by affecting motility and biofilm formation capacity of the bacteria, by attacking cell membranes or by exhibiting unknown toxic effects remains to be investigated. Nevertheless, the fact that they are important in oral infections but, at least in case of orfamide, not when bacteria are directly injected into the hemocoel, supposes an action

during the gut stage of infection. Virulence of mutants that are deficient for the production of several other antimicrobial compounds, i.e. phenazines, 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin and toxoflavin, was not attenuated (Loper *et al.*, 2016)(Chapter 3). However, *P. protegens* CHA0 was expressing the biosynthesis genes for 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin during the infection of insects (Chapter 3), which suggests that these metabolites still might serve a function during colonization or killing of the insect. Under natural conditions antimicrobial metabolites might be of relevance to compete with microbes of the resident midgut flora and thereby would contribute to persistence. In general, factors contributing to persistence in the gut, might not be unique to insecticidal strains, since also strains which were not able to kill insects were found to persist in *Spodoptera littoralis* larvae (Chapter 2).

Although the above-mentioned enzymes and cyclic lipopeptides may contribute to insect virulence in the gut, they are not oral toxins which would cause mortality on their own. In contrast, the authors of a very recent publication discovered an insect toxin in a *P. chlororaphis* strain, which exhibits efficient oral activity against corn rootworms (Schellenberger *et al.*, 2016). The protein IPD072Aa applied as crude extract or produced by transgenic corn plants is able to kill *Diabrotica* larvae without the need of additional factors or living bacteria. This toxin seems to exhibit a strong host specificity and is not active against several lepidopteran and hemipteran larvae (Schellenberger *et al.*, 2016)(Table 1). Interestingly, we found homologues of IPD072Aa also in the genomes of several of the strains studied in Chapter 2. A protein of *P. chlororaphis* PCL1391, encoded by locus tag PCL1391_1896, shares 88% amino acid identity (aai) with IPD072Aa and comparably high aai were detected for most of the other *P. chlororaphis* strains (data not shown). Therefore, it will be interesting to assess the contribution of these toxins to insecticidal activity of our strains. However, the toxin seems not to be universal to the *P. chlororaphis* subgroup. In the genome of *P. protegens* Pf-5 a protein with 36% aai was detected (Schellenberger *et al.*, 2016), but the genome of *P. protegens* CHA0 does not seem to harbor a homologue (data not shown).

Once the bacteria have reached the hemocoel, Fit might come into action. While transgenic expression of *fitD* in *Escherichia coli* was sufficient to render this bacterium highly toxic when injected into insect larva (Péchy-Tarr *et al.*, 2008), it did not cause significant mortality in oral infections (Ruffner *et al.*, 2013). Furthermore, Fit was found

Table 1 Factors contributing to insecticidal activity of *Pseudomonas chlororaphis* subgroup bacteria

Factor	Strain	Method of application	Effect observed in	No effect observed in	Reference
Fit	CHA0	injection	*Galleria mellonella* *Manduca sexta*		Péchy-Tarr *et al.* (2008)
		oral	*Spodoptera littoralis*		Ruffner *et al.* (2013)
	Pf-5	injection	*G. mellonella* *M. sexta*		Péchy-Tarr *et al.* (2008)
		oral	*M. sexta*	*Drosophila melanogaster*	Rangel *et al.* (2016) Loper *et al.* (2016)
	PCL1391	oral	*S. littoralis*		Ruffner *et al.* (2013)
Chitinase C	CHA0	injection		*G. mellonella*	Chapter 2
		oral	*Plutella xylostella*		Chapter 2
	Pf-5	oral	*D. melanogaster*		Loper *et al.* (2016)
Phospho-lipase C	CHA0	injection		*G. mellonella*	Chapter 2
		oral	*P. xylostella (minor)*		Chapter 2
Hydrogen cyanide	CHA0	injection	*G. mellonella*		Chapter 3
		oral	*P. xylostella (minor)*		Chapter 3
		Contact (live cells)	*Odontotermes obesus*		Devi and Kothamasi (2009)
	Pf-5	oral		*D. melanogaster*	Loper *et al.* (2016)
	PCL1391	injection	*G. mellonella*		Chapter 3
		oral		*P. xylostella*	Chapter 3
Rhizoxin	Pf-5	oral	*D. melanogaster*		Loper *et al.* (2016)
Orfamide	CHA0	injection		*G. mellonella*	Chapter 3
		oral	*P. xylostella*		Chapter 3
	Pf-5	oral	*D. melanogaster*		Loper *et al.* (2016)
	CMR12a	injection		*G. mellonella*	Chapter3
		oral	*P. xylostella (minor)*		Chapter 3
	F6	Contact (purified metabolite)	*Myzus persicae*		Jang *et al.* (2013)
Clp1391	PCL1391	injection	*G. mellonella*		Chapter 3
		oral	*P. xylostella (minor)*		Chapter 3
Sessilin	CMR12a	injection	*G. mellonella*		Chapter 3
		oral	*P. xylostella*		Chapter 3
LPS	CHA0	injection	*G. mellonella*		Kupferschmied *et al.* (2016)
		oral	*P. xylostella*		
	PCL1391	injection		*G. mellonella*	
IPD072Aa	*P. chloro-raphis*	Oral (protein)	*Diabrotica virgifera* *Diabrotica barberi*	*A.i.*, *H.z.*, *O.n.*, *S.f.*, *P.i.*, *L.h.*	Schellenberger *et al.* (2016)
Type VI secretion apparatus	Pf-5	oral		*D. melanogaster*	Loper *et al.* (2016)
	CHA0	oral	*Pieris brassicae*		Keel (2016) Péchy-Tarr *et al.* (unpublished)
Exopoly-saccharides	CHA0	oral	*P. brassicae*		Keel (2016) Terrattaz *et al.* (unpublished)
Regulator					
GacS/ GacA	CHA0	injection		*G. mellonella*	Chapter 3
		oral	*S. littoralis*		Ruffner *et al.* (2013)
			P. xylostella		Chapter 2
	Pf-5	oral	*D. melanogaster*		Olcott *et al.* (2010)
	PCL1391	injection	*G. mellonella*		Chapter 3
		oral	*P. xylostella*		Chapter 3
PhoP-PhoQ			*G. mellonella*		Kupferschmied (2015)

LPS, lipopolysaccharide; CHA0, *Pseudomonas protegens* CHA0; Pf-5, *Pseudomonas protegens* Pf-5; PCL1391, *Pseudomonas chlororaphis* PCL1391; CMR12a, *Pseudomonas* sp. CMR12a; *A.i.*, *Agrotis ipsilon*; *H.z.*, *Helicoverpa zea*; *O.n.*, *Ostrinia nubilalis*; *S.f.*, *Spodoptera frugiperda*; *P.i.*, *Pseudoplusia includes*; *L.h.*, *Lygus hesperus*

to be specifically produced in the insect hemolymph (Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014) and is only rarely and weakly expressed at early time points of oral infections (Chapter 3). Taken together, these findings indicate that Fit exhibits a direct toxic effect in the hemolymph, but not in the gut. An additional factor presumably acting in the hemolymph is hydrogen cyanide, which was found to contribute to injectable activity of *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (Chapter 3) and which can interfere with the mitochondrial respiratory chain (Way, 1984; Devi and Kothamasi, 2009).

For successful infection, bacteria have to overcome or escape the insect's immune system. First evidence for the occurrence of strategies to evade the immune system come from a recent study by Kupferschmied *et al.* (2016), which showed that the insecticidal strains of the *P. fluorescens* group (as identified in Chapter 2), but not the non-insecticidal strains (with one exception) are resistant to polymyxin B, which is indicative for resistance to insect antimicrobial peptides. Indeed, *P. protegens* CHA0 was found to be resistant against the insect antimicrobial peptides cecropin A and B, too. The authors further demonstrated that the structure of the lipopolysaccharides (LPS), the major cell surface component of Gram-negative bacteria, is decisive for antimicrobial peptide resistance. *P. protegens* CHA0 lacking its dominant form of O-antigenic polysaccharide, the exposed part of the LPS, exhibited reduced virulence towards *Galleria mellonella* and *Plutella xylostella* larvae upon injection or feeding, respectively, and showed reduced ability to colonize *G. mellonella* (Kupferschmied *et al.*, 2016). Thus, specific modifications of cell surface components may protect insecticidal pseudomonads from the insect immune response. Besides LPS also other cell-surface exposed components in particular certain exopolysaccharides may contribute to insect toxicity (Keel, 2016).

In general, there is evidence for the existence of general pathogenicity factors common to all *P. chlororaphis* subgroup strains (Chapter 2) but also for strain specific factors. One of them is rhizoxin produced by Pf-5 (Table 1), the action of which is still speculative but seems to be correlated with the observation of eye defects in *D. melanogaster* (Loper *et al.*, 2016). A type VI secretion system was found not to be crucial for oral infections of *D. melanogaster* by *P. protegens* Pf-5 (Loper *et al.*, 2016); however, it seems to contribute to virulence of *P. protegens* CHA0 (Keel, 2016).

In the final step of infection, when the insect surrenders to septicemia, factors that favor nutrient acquisition and degradation of the dead insect (e.g. chitinases) and that

antagonize other microbes colonizing the cadaver (e.g all the antimicrobial compounds) might be of importance. In accordance, genes required for antimicrobial metabolite production of *P. protegens* CHA0 were found to be expressed in dead larvae (Chapter 3).

In summary, although the number of known pathogenicity factors drastically increased within the last year, their mode of action is still speculative. Microscopic examination of the infection process (Chapter 4) may help to understand how *P. chlororaphis* subgroup/sub-clade 1 bacteria infect their hosts and could be used to study the behavior of mutants lacking specific factors during the course of infection. However, host specific differences in infections and the existence of strain-specific toxicity factors make insecticidal activity of *P. chlororaphis* subgroup bacteria a very complex matter.

Furthermore, moderate insecticidal activity has been discovered in strains of the *P. fluorescens subgroup*/sub-clade 3 (Olcott *et al.*, 2010; Rangel *et al.*, 2016) (Chapter 2), but to date not a single pathogenicity factor involved in their virulence has been identified. Since these strains lack several important factors present in sub-clade 1, such as Fit, chitinase C, or phospholipase C, they might rely on completely different mechanisms, which could represent an intriguing field for future research.

5.3. Ecological considerations – the insect as alternative host

While more and more mechanisms and factors involved in insecticidal activity of *P. fluorescens* group bacteria are getting discovered (Table 1), we are only at the very beginning of gaining an insight into the ecological significance of this trait. Observations of successful infections of laboratory-reared model insects do not allow conclusions on the situation in nature. Already under controlled conditions of lab experiments, the pathogenicity of a given strain can vary considerably and is dependent on manifold factors, such as humidity, light conditions, insect diet or other stress factors (e.g. starvation of larvae before exposure to bacteria-inoculated diet). In agricultural soils, natural root-colonization by *P. chlororaphis* subgroup bacteria is probably often not higher than 10^5 cfu per g of roots (F. Dennert, personal communication). Hence, insects feeding on roots might not ingest bacterial numbers comparable to those applied in virulence assays and the internalized bacteria would encounter a natural midgut flora to compete with. Although causing high mortality rates in lab experiments, the extent of virulence exhibited by *P. chlororaphis* subgroup bacteria under natural conditions is

therefore still unclear and it might well be, that these bacteria are rather opportunistic pathogens.

In experiments with *Delia radicum* and *Otiorhynchus sulcatus* (Chapter 4), which were performed under more natural conditions than earlier studies, *P. protegens* CHA0 was able to persist in the insects but did not cause significant mortality. The persistence throughout different developmental stages provides the prerequisite for insect-mediated dispersal of *P. protegens* CHA0 not only upon ingestion by larvae, but also by the adult stage (Figure 1). This is of significant ecological impact, since adult insects are more mobile than their larval stage and cover larger distances, especially in search of new host plants for egg deposition. Our experiment with *D. radicum* provides the first evidence that *P. protegens* CHA0 can use insect hosts as a means for dispersal. Transstadial transmission of CHA0 was also found in *O. sulcatus* and therefore adult weevils are presumably also able to transmit the bacteria to new host plants. In general, vectoring of plant-beneficial rhizobacteria by root-feeding insects might be a frequent incident, the investigation of which, however, has been neglected until now. Insecticidal activity is only found in the *P. chlororaphis* subgroup/ sub-clade 1 and as a mild form in certain strains of the *P. fluorescens* subgroup/ sub-clade 3 (Chapter 2); on the contrary, persistence is also found in sub-clade 2 strains, which are not able to kill insects (Chapter2). Hence, also for these strains, insects might have an ecological role as vectors.

We provide a proof of principle for insect-mediated dispersal of plant-beneficial rhizobacteria, but the exact mechanisms, the localization in or on the insect host, and the relevance of this behavior in nature remain to be investigated. If the bacteria are using the insect host for dispersal, attenuated virulence might be of advantage, thus causing the insect to survive long enough to ensure dispersal of bacteria to a distant location. Nevertheless, also in nature, individual insects can be injured or weakened, because of biotic or abiotic stresses. In these cases, *P. chlororaphis* subgroup bacteria might take their chance, invade the insect hemocoel and use it as a vessel for mass replication. Once the nutritious insect cadaver has been consumed, the millions of resulting bacteria will spread and recolonize plant roots (Figure 1).

The colonization of two alternative hosts of different kingdoms, plant and insect, can cause an evolutionary struggle for bacteria, since certain factors that are advantageous in one host may be unfavorable in the other (Nadarasah and Stavrinides, 2011). This trade-off may be reduced by host-specific production of costly proteins or

metabolites. The host specific expression of *fitD* in the insect but not on plant roots (Péchy-Tarr *et al.*, 2013; Kupferschmied *et al.*, 2014) implies that such a behavior may indeed occur in fluorescent pseudomonads.

Concerning the plant host, *P. protegens* CHA0 is a generalist, colonizing the roots of monocotyledonous and dicotyledonous species (Keel *et al.*, 1992; Maurhofer *et al.*, 1994; Siddiqui *et al.*, 2006). The colonization and especially the killing of the insect seem to be more variable between different host species. While CHA0 for instance exhibited strong oral activity against different lepidopteran species and against aphids, it did not cause mortality in bumblebees, mosquitos or cabbage maggots (Ruffner, 2013; Kupferschmied, 2015)(Chapter 4). However, to define the true host range of *P. protegens* CHA0 is difficult, because different insect species have been tested under different conditions and from experience we know that experimental conditions can be decisive whether *P. protegens* CHA0 is able to kill insect larvae or not (data not shown).

For the outcome of infection not only the insect species matters, but also the bacterial strain. Different strains of the *P. chlororaphis* subgroup exhibited differential effectiveness in killing larvae of *P. xylostella* and *G. mellonella*, though all strains caused high mortality rates (Chapter 2). Against *D. melanogaster* larvae only strain *P. protegens* Pf-5 was found to be highly virulent, but not the two *P. chlororaphis* strains 06 and 30-84 (Rangel *et al.*, 2016) and neither was *P. protegens* CHA0 (Binggeli, 2009). In line with this, a *P. chlororaphis* strain of unknown subspecies, or at least its toxin, exhibits a host specificity for corn rootworms (Schellenberger *et al.*, 2016).

To date, research on insecticidal activity of fluorescent pseudomonads has mostly concentrated on root isolates with known plant-beneficial traits. To understand ecological aspects of insecticidal activity in the *P. fluorescens* group, the natural occurrence of these bacteria has to be investigated, for instance, by especially looking for these bacteria in insect-associated communities. By extraction of different arthropods, already several strains belonging to the *P. chlororaphis* subgroup could be isolated, which gives a first indication that insects represent indeed a natural host/niche for these bacteria (P. Vesga and J. Schneider, personal communication). Furthermore, we might have to broaden our view and even include habitats beyond plants and insects. The genus *Pseudomonas* is extremely versatile, harboring pathogens, beneficials, and saprophytes, and the *P. fluorescens* group might inhabit many more niches than we are currently aware of. This hypothesis is supported by the recent isolation of *P. chlororaphis* subsp. *piscium*

DSM21509T from perch (Burr *et al.*, 2010) and *P. protegens* BRIP and *P. chlororaphis* CD from cyclops (Ruffner *et al.*, 2015), suggesting water environments and their inhabitants, even vertebrates, as potential habitats. Finally, the finding that many strains are performing well on plants and in insects, independently of their origin (Chapter 2), suggests that these bacteria are truly multi-talents, easily able to switch between different niches and providing us with many more facets to explore.

5.4. Potential of insecticidal pseudomonads for biocontrol of insect pests

This thesis provides considerable knowledge in terms of ecological aspects and the identification of additional virulence factors but it gives only fist indications about the biocontrol potential of insecticidal fluorescent pseudomonads (Chapter 4). A test-system with cauliflower plants and the root pest *D. radicum* was developed and will provide a useful tool to study the activity of biocontrol agents against this insect species in the future. However, under the tested conditions *P. protegens* CHA0 did not affect survival of *D. radicum* larvae; still, morphological defects suggest that adverse effects caused by the bacteria may exist (Chapter 4). Considering the results of experiments with another dipteran species, *D. melanogaster*, strain CHA0 might not have been the best choice to test for effects against *D. radicum*. While *P. protegens* Pf-5 harbors rhizoxin and efficiently kills *D. melanogaster*, *P. protegens* CHA0 does not, a finding, which might also apply to other dipteran species (Binggeli, 2009; Olcott *et al.*, 2010; Loper *et al.*, 2016). Therefore, repeating the experiment with strain Pf-5 or its close relative PF, might allow the detection of a biocontrol effect.

Encouraging results regarding biocontrol effects against root pests come from recent field experiments. In corn fields, which were artificially infested with eggs of the western corn rootworm, significantly less larvae could be recovered from plants that had been inoculated with a mixture of *P. chlororaphis* PCL1391 and *P. protegens* CHA0 than from control plants. In addition, larval size in the bacterial treatment was significantly reduced (N. Imperiali and G. Jaffuel, personal communication). Furthermore, the recent report on oral activity of the *P. chlororaphis* toxin IPD072Aa against corn rootworms suggests a true potential for biocontrol of root pests by *P. chlororaphis* subgroup bacteria. However, since there seems to be a strain-dependent host specificity, for each target pest the most effective strain(s) have to be identified.

Beside their direct insecticidal activity, fluorescent pseudomonads could also be interesting for plant protection due to their ability to induce systemic resistance (Figure 1). In field experiment, wheat plants inoculated with *P. protegens* CHA0 or *P. chlororaphis* PCL1391 or both were much less affected by a naturally occurring infestation with *Oscinella frit*, a fly that causes damage by boring into the shoots (N. Imperiali and F. Dennert, personal communication). The topic of induced systemic resistance was also touched during this thesis, in the frame of which projects of master and bachelor students studying this phenomenon were co-supervised. While no clear effect of root-colonization by *P. protegens* CHA0 against leaf-feeding *S. littoralis* larvae on *Arabidopsis thaliana* could be detected (Fataar, 2013), testing selected strains (out of the 26 strains used in Chapter 2) against the leaf-feeders *Pieris brassicae* and *Spodoptera exigua* on *Brassica napus* plants (Grandi, 2014) or against the sucking insect *Aphis gossypii*, (Dalbosco, 2015), gave first indications for the occurrence of induced systemic resistance against insects and revealed interesting candidate strains for future studies.

5.5. Perspectives and concluding remarks

This thesis yielded some first answers to our initial research questions dealing with the identification of virulence factors, the role of the insect as an alternative host and a means for dispersal as well as the biocontrol potential of fluorescent pseudomonads against insect pests. It also generated tools, which will prove beneficial for further studies on this topic. Hence, the feeding assay established with *P. xylostella* (Chapter 2 and 3) can be further used to study virulence factors, virulence regulation and the course of infection, while the *Delia*-cauliflower experiment (Chapter 4) can be used to explore ecological and biocontrol aspects in more detail. Furthermore, the sequencing and assembly of twenty-four genomes belonging to strains throughout the entire *P. fluorescens* group (Chapter 2) represents an extremely useful basis for future investigations. These genomes provide, for instance, a tool to study evolutionary events, to assess phylogenetic relationships with the possibility to reclassify strains currently endued with wrong species names, to search for further insecticidal factors, and to explore the genetic basis of any trait exhibited by the group of sequenced strains. Hitherto, the genomes already allowed to study the diversity of O-polysaccharide biosynthesis genes within the *P. fluorescens* group (Kupferschmied *et al.*, 2016). O-polysaccharides were demonstrated to be involved in

insecticidal activity and the diversity of their structures was hypothesized to reflect the adaption to different niches (Kupferschmied *et al.*, 2016). *P. chlororaphis* and *P. protegens* seem to be able to switch between a plant- and an insect-associated lifestyle; however, we merely understand how these bacteria are able to do so. Comparing bacterial gene expression on roots and in insects might reveal some insights. The about 200 genes identified in Chapter 2 to be specific for insecticidal strains might be of special interest in terms of expression in the insect host.

The discovery of insecticidal activity in root-colonizing plant beneficial bacteria nourished the visions on possible applications of these bacteria. Their dual activity against plant diseases and insect pests opens the possibility of a combined application against both threats in parallel. To obtain optimal biocontrol effects against fungal diseases and/or insect pests, a consortium-based approach might be most promising, since several studies report on enhanced biocontrol effects of mixtures of biocontrol organisms, compared to the respective single treatments (Koppenhofer and Kaya, 1997; Raupach and Kloepper, 1998; Broderick *et al.*, 2000; Thomas *et al.*, 2003; Wraight and Ramos, 2005; Raymond *et al.*, 2008; Grosch *et al.*, 2012). Combining different bacterial strains with complementary features or insect host spectra may result in broader and more robust crop protection. In addition, using several strains with differing virulence mechanisms may prevent the development of resistance in the target pest. Even though biocontrol is assumed to be less prone to evolution of resistance than pesticides, the application of a single strain over large areas and in high amounts may generate enough selective pressure for resistance to develop (Bardin *et al.*, 2015). Besides strain combinations, also combinations of insecticidal pseudomonads with other entomopathogenic organisms, such as fungi or nematodes, should be envisaged. Since *P. chlororaphis* subgroup bacteria need to reach the hemocoel to cause fatal infections, assisted-entry or weakening of the insect by fungi or nematodes might lead to higher *Pseudomonas* infection rates. Nevertheless, the prerequisite for a successful application of consortia is the right choice of organisms, since these can also negatively affect each other.

Biocontrol products such as Cedomon (Mark *et al.*, 2006) or Proradix (Buddrus-Schiemann *et al.*, 2010) available on the market against root diseases may also work against insect pests, since Cedomon is based on a *P. chlororaphis* strain and Proradix was found to exhibit insecticidal activity against *G. mellonella* (data not shown). Beside control

of root pests, also a foliar application is conceivable, since major leaf pests such as *S. littoralis* or *P. xylostella* were found to be highly susceptible to *P. chlororaphis* and *P. protegens* (Ruffner *et al.*, 2013)(Chapter 2). To this end, strains with good survival capacities in the phyllosphere or formulations prolonging survival on leaves would be needed. Moreover, individual insect toxins instead of entire strains can be exploited for pest control, as it was recently demonstrated by the generation of transgenic corn plants expressing a *P. chlororaphis* insect toxin (Schellenberger *et al.*, 2016). In any case more research on mechanisms underlying insecticidal activity and determining the host-spectrum of different strains as well as field trials will be crucial. For a safe introduction of fluorescent pseudomonads as biocontrol agents against insect, we should further gain a better understanding of their ecology.

In summary, the work presented in this thesis generated answers to several questions on insecticidal activity of fluorescent pseudomonads, but, as often in research, finding one answer results in the emergence of a multitude of new questions and hypotheses. Research on insecticidal activity of plant-beneficial pseudomonads is still in its infancy and many interesting aspects are still awaiting their discovery.

5.6. References

Bardin M., Ajouz S., Comby M., Lopez-Ferber M., Graillot B., Siegwart M. *et al.* (2015). Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? *Frontiers in plant science* 6**:** 566.

Binggeli O. (2009). Molecular analysis of insecticidal activity in the plant-beneficial bacterium *Pseudomons fluorescens.* Master thesis, University of Lausanne, Lausanne, Switzerland.

Broderick N. A., Goodman R. M., Raffa K. F., Handelsman J. (2000). Synergy between zwittermicin A and *Bacillus thuringiensis* subsp. *kurstaki* against gypsy moth (Lepidoptera: Lymantriidae). *Environ Entomol* 29**:** 101-107.

Buddrus-Schiemann K., Schmid M., Schreiner K., Welzl G., Hartmann A. (2010). Root Colonization by Pseudomonas sp. DSMZ 13134 and Impact on the Indigenous Rhizosphere Bacterial Community of Barley. *Microb Ecol* 60**:** 381-393.

Burr S. E., Gobeli S., Kuhnert P., Goldschmidt-Clermont E., Frey J. (2010). *Pseudomonas chlororaphis* subsp. *piscium* subsp. nov., isolated from freshwater fish. *Int J Syst Evol Microbiol* 60**:** 2753-2757.

Dalbosco A. (2015). Effects of phylogenetically diverse plant-associated fluorescent pseudomonads on activation of induced systemic resistance against fungi, insects and viruses in cucumber. Bachelor thesis, ETH Zurich, Zurich, Switzerland.

De Vleesschauwer D., Höfte M. (2009). Rhizobacteria-induced systemic resistance. *Adv Bot Res* 51**:** 223-281.

Devi K. K., Kothamasi D. (2009). *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting cytochrome *c* oxidase of the termite respiratory chain. *FEMS Microbiol Lett* 300**:** 195-200.

Fataar S. (2013). Plant-mediated effects in insect - rhizobacteria interactions. Master thesis, ETH Zurich, Zurich, Switzerland.

ffrench-Constant R., Dowling A. (2014). *Photorhabdus* toxins. In: Dhadialla T. S., Gill S. S. (eds). *Advances in Insect Physiology*. vol. 47. Elsevier: London, UK. pp 343-388.

Grandi L. (2014). Effects of insecticidal and non-insecticidal fluorescent pseudomonad root colonization on plant resistance and volatile production. Master studies, research project thesis, ETH Zurich, Zurich, Switzerland.

Grosch R., Dealtry S., Schreiter S., Berg G., Mendonca-Hagler L., Smalla K. (2012). Biocontrol of *Rhizoctonia solani*: complex interaction of biocontrol strains, pathogen and indigenous microbial community in the rhizosphere of lettuce shown by molecular methods. *Plant Soil* 361**:** 343-357.

Haas D., Défago G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 3**:** 307-319.

Ikezawa H., Hashimoto A., Taguchi R., Nakabayashi T., Himeno M. (1989). Release of PI-anchoring enzymes and other effects of phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* on TN-368 cells from a moth ovary. *Toxicon* 27**:** 637-645.

Jang J. Y., Yang S. Y., Kim Y. C., Lee C. W., Park M. S., Kim J. C. *et al.* (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J Agric Food Chem* 61**:** 6786-6791.

Jurat-Fuentes J. L., Jackson T. A. (2012). Bacterial entomopathogens. In: Vega F. E., Kaya H. K. (eds). *Insect Pathology*, 2 edn. Elsevier: London, UK. pp 265-349.

Keel C., Schnider U., Maurhofer M., Voisard C., Laville J., Burger U. *et al.* (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0 - Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant-Microbe Interact* 5**:** 4-13.

Keel C. (2016). A look into the toolbox of multi-talents: insect pathogenicity determinants of plant-beneficial pseudomonads. *Environ Microbiol* 18**:** 3207-3209.

Koppenhofer A. M., Kaya H. K. (1997). Additive and synergistic interaction between entomopathogenic nematodes and *Bacillus thuringiensis* for scarab grub control. *Biol Control* 8**:** 131-137.

Kupferschmied P., Péchy-Tarr M., Imperiali N., Maurhofer M., Keel C. (2014). Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. *PLoS Path* 10**:** e1003964.

Kupferschmied P. (2015). Molecular basis and regulation of insect pathogenicity in plant-beneficial pseudmonads. PhD thesis, University of Lausanne.

Kupferschmied P., Chai T., Flury P., Blom J., Smits T. H., Maurhofer M. *et al.* (2016). Specific surface glycan decorations enable antimicrobial peptide resistance in plant-beneficial pseudomonads with insect-pathogenic properties. *Environ Microbiol* [Epub ahead of print].

Loper J. E., Henkels M. D., Rangel L. I., Olcott M. H., Walker F. L., Bond K. L. *et al.* (2016). Rhizoxin, orfamide A, and chitinase production contribute to the toxicity of *Pseudomonas protegens* strain Pf-5 to *Drosophila melanogaster*. *Environ Microbiol* 18**:** 3509-3521.

Lysenko O. (1972). Pathogenicity of *Bacillus cereus* for insects II. Toxicity of phospholipase C for *Galleria mellonella*. *Folia Microbiol* 17**:** 228-&.

Mark G. L., Morrissey J. P., Higgins P., O'Gara F. (2006). Molecular-based strategies to exploit *Pseudomonas* biocontrol strains for environmental biotechnology applications. *FEMS Microbiol Ecol* 56**:** 167-177.

Maurhofer M., Keel C., Haas D., Defago G. (1994). Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber. *Eur J Plant Pathol* 100**:** 221-232.

Nadarasah G., Stavrinides J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol Rev* 35**:** 555-575.

Olcott M. H., Henkels M. D., Rosen K. L., Walker F. L., Sneh B., Loper J. E. *et al.* (2010). Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. *PloS one* 5**:** e12504.

Péchy-Tarr M., Bruck D. J., Maurhofer M., Fischer E., Vogne C., Henkels M. D. *et al.* (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ Microbiol* 10**:** 2368-2386.

Péchy-Tarr M., Borel N., Kupferschmied P., Turner V., Binggeli O., Radovanovic D. *et al.* (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ Microbiol* 15**:** 736-750.

Rangel L. I., Henkels M. D., Shaffer B. T., Walker F. L., Davis E. W., 2nd, Stockwell V. O. *et al.* (2016). Characterization of toxin complex gene clusters and insect toxicity of bacteria representing four subgroups of *Pseudomonas fluorescens*. *PloS one* 11**:** e0161120.

Raupach G. S., Kloepper J. W. (1998). Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88**:** 1158-1164.

Raymond B., Lijek R. S., Griffiths R. I., Bonsall M. B. (2008). Ecological consequences of ingestion of *Bacillus cereus* on *Bacillus thuringiensis* infections and on the gut flora of a lepidopteran host. *J Invertebr Pathol* 99**:** 103-111.

Regev A., Keller M., Strizhov N., Sneh B., Prudovsky E., Chet I. *et al.* (1996). Synergistic activity of a *Bacillus thuringiensis* delta-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Appl Environ Microbiol* 62**:** 3581-3586.

Ruffner B. (2013). Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis, ETH Zurich, Zurich, Switzerland.

Ruffner B., Péchy-Tarr M., Ryffel F., Hoegger P., Obrist C., Rindlisbacher A. *et al.* (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ Microbiol* 15**:** 751-763.

Ruffner B., Péchy-Tarr M., Höfte M., Bloemberg G., Grunder J., Keel C. *et al.* (2015). Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* 16**:** 609.

Sampson M. N., Gooday G. W. (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiol-UK* 144**:** 2189-2194.

Schellenberger U., Oral J., Rosen B. A., Wei J.-Z., Zhu G., Xie W. *et al.* (2016). A selective insecticidal protein from *Pseudomonas* for controlling corn rootworms. *Science* [Epub ahead of print].

Siddiqui I. A., Shaukat S. S., Sheikh I. H., Khan A. (2006). Role of cyanide production by *Pseudomonas fluorescens* CHA0 in the suppression of root-knot nematode, *Meloidogyne javanica* in tomato. *World J Microbiol Biotechnol* 22**:** 641-650.

Thomas M. B., Watson E. L., Valverde-Garcia P. (2003). Mixed infections and insect-pathogen interactions. *Ecol Lett* 6**:** 183-188.

Way J. L. (1984). Cyanide intoxication and its mechanism of antagonism. *Annu Rev Pharmacol Toxicol* 24**:** 451-481.

Wraight S. P., Ramos M. E. (2005). Synergistic interaction between *Beauveria bassiana*- and *Bacillus thuringiensis tenebrionis*-based biopesticides applied against field populations of Colorado potato beetle larvae. *J Invertebr Pathol* 90**:** 139-150.

ACKNOWLEDGMENT

This PhD thesis would not have been possible without the many people who gave me support, advice and guidance throughout the time I was working on it. Therefore, I want to thank:

My supervisors, Dr. Monika Maurhofer and Dr. Christoph Keel, for their great support while doing this project, giving me freedom and guidance at the same time. Monika, your never-ending energy and enthusiasm is impressive and inspiring. You take care of people in all respects and really deserve the title 'Doktormutter'.

Prof. Dr. Bruce McDonald for his support and for hosting me in this wonderful group.

Dr. Philipp Engel for agreeing to be my co-examiner. Thank you for your time.

Dr. Theo Smits for his patience in introducing me into the world of genome assembly and genomics.

Dr. Regina Kleespies, Prof. Dr. Cornelia Ullrich, Dr. Astrid Keller and Helga Radke for the fascinating hours behind the microscope. I really enjoyed them a lot.

All the students and other helpers who supported me in the lab; especially, Nora, Shakira, Sandra, Ana, Dylan, Anja and Nicolas. I would never have been able to do this project without your help!

Current and former members of the plant-pathology group for providing a wonderful working atmosphere and especially for all the good times on excursions, ski week-ends, insti-parties, barbecues, international food festivals and so on, but also during everyday life at coffee-breaks, lunches, in the lab and the office.

My friends and office mates Andrea, Zane, Beat, Francesca, Pili and Tobi for discussions, support, relaxing hours during yoga and also just their company.

The Keel lab, especially Peter for fruitful discussions and for hosting me at his place during my stays in Lausanne and Maria for her great supervision in the lab and for the generation of mutants.

People at JKI Darmstadt for the great time at their institute and for all the memorable hours we had together at conferences.

Ueli and Marcello for their never-ending technical assistance, Sandra and Uli for their great support with all sorts of administrative things and Elizabeth for all the cleaning work and wonderful cakes

The ETH staff of the Genetic Diversity Centre (GDC) for their laboratory support.

The Swiss National Science Foundation for founding this work.

The ‘Fix the leaky pipline’- women and all the other brilliant women scientists, who I met during the work on this thesis and who inspired me a lot while working in this still men-dominated world of science.

Florian, Andrea, and Lea for constructive criticism on the manuscript of this thesis.

And last, but not least:

My friends inside and outside the lab for being there and cheering me up, when things weren’t running as they were expected to.

My family for their huge support; especially Regine, Richi, Kathrin and Felix for the countless numbers of hours taking care of Lisa. Florian for his amazing support in every respect and Lisa for great moments of joy during breaks of thesis writing and just for being such a wonderful, cheerful and open-hearted baby, which made it to me as easy to finish this thesis as it can be as a mother.

SCIENTIFIC CONTRIBUTIONS

Published work

Kupferschmied P., Chai T., **Flury P.**, Blom J., Smits T. H., Maurhofer M. *et al.* (2016). Specific surface glycan decorations enable antimicrobial peptide resistance in plant-beneficial pseudomonads with insect-pathogenic properties. *Environ Microbiol* 10.1111/1462-2920.13571**:** [Epub ahead of print].

Flury P., Aellen N., Ruffner B., Pechy-Tarr M., Fataar S., Metla Z. *et al.* (2016a). Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics. *The ISME journal* 10**:** 2527-2542.

Klauser D., Desurmont G. A., Glauser G., Vallat A., **Flury P.**, Boller T. *et al.* (2015). The *Arabidopsis* Pep-PEPR system is induced by herbivore feeding and contributes to JA-mediated plant defence against herbivory. *J Exp Bot* 66**:** 5327-5336.

Lori M., van Verk M. C., Hander T., Schatowitz H., Klauser D., **Flury P.** *et al.* (2015). Evolutionary divergence of the plant elicitor peptides (Peps) and their receptors: interfamily incompatibility of perception but compatibility of downstream signalling. *J Exp Bot* 66**:** 5315-5325.

Klauser D., **Flury P.**, Boller T., Bartels S. (2013). Several MAMPs, including chitin fragments, enhance AtPep-triggered oxidative burst independently of wounding. *Plant signaling & behavior* 8**:** e25346.

Flury P., Klauser D., Boller T., Bartels S. (2013). MAPK phosphorylation assay with leaf disks of *Arabidopsis Bio-protocol* 3**:** e929.

Flury P., Klauser D., Schulze B., Boller T., Bartels S. (2013). The anticipation of danger: microbe-associated molecular pattern perception enhances *At*Pep-triggered oxidative burst. *Plant Physiol* 161**:** 2023-2035.

Boller T. & **Flury P.** (2012). Peptides as danger Signals: MAMPs and DAMPs. In: Irving H. R., Gehring C. (eds). *Plant signaling peptides*. Springer: Heidelberg, Germany. pp 163-181.

Unpublished work

Flury P., Vesga P., Péchy-Tarr M., Aellen N., Dennert F., Hofer N. *et al.* (2016b). Antimicrobial and insecticidal: cyclic lipopeptides and hydrogen cyanide produced by plant-beneficial *Pseudomonas* strains CHA0, CMR12a and PCL1391 contribute to insect killing. submitted to *Front Microbiol*., accepted with minor revisions, 19th October 2016

Klauser D., **Flury P.**, Boller T., Bartels S. (2016). Looking BAK again: Is an old acquaintance of innate immunity involved in the detection of herbivores. *Plant signaling & behavior***:** in press.

Flury P., Vesga P., Dominguez-Ferreras A., Bär D., Tinguely C., Schneider J. *et al.* The plant-beneficial root-colonizer *Pseudomonas protegens* CHA0 persists in insects throughout different developmental stages and can be transmitted to new host plants**.** Manuscript in preparation.

Conferences and Presentations

2015

ZHAW-SIB Summer School GEMP 2015 – Genomics and Evolution of Microbial Pathogens (24.8.-04.09.2015, Wädenswil, Switzerland)
Guest lecture: Insect pathogenicity in plant-beneficial pseudomonads: Phylogenetic distribution and comparative genomics

15th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests" (07.-11. June 2013, Riga, Latvia)
Oral presentation: What are the features that plant-beneficial pseudomonads require to become insect pathogens?

10th International PGPR Workshop 2015 (16.-19.06.2015, Liège, Belgium)
Oral presentation: What are the features that plant-beneficial pseudomonads require to become insect pathogens?

2014

DMF Impromptu (07.11.2014, UNIL, Lausanne, Switzerland)
Participation without contribution

59. Deutsche Pflanzenschutztagung (23.-26. September 2014, Freiburg, Germany)
Oral presentation: Insecticidal activity in root-colonizing plant-beneficial pseudomonads

International congress on invertebrate pathology and microbial control 2014 (03.-08.08.2014, Mainz, Germany)
Oral presentation: Untangling insect pathogenicity in plant-beneficial pseudomonads: a combination of comparative genomics, bioassays and histopathology

Institute seminary, Julius-Kühn Institute, Institute for Biological Control (9.1.2013, Darmstadt, Germany)
Oral presentation: The secret life of plant-beneficial pseudomonads: from roots to insect guts

2013

Autumn meeting of the Swiss Society of Phytiatry (26.09.2013, Nyon, Switzerland)
Poster: "Untangling insect pathogenicity in plant-associated pseudomonads by a combination of comparative genomics and bioassays"

14th International Conference on *Pseudomonas* (7.-11.09.2013, Lausanne, Switzerland)
Poster: "Untangling insect pathogenicity in plant-associated pseudomonads by a combination of comparative genomics and bioassays"

IOBC-WPRS Working Group “Insect Pathogens and Insect Parasitic Nematodes” (16.-20. June 2013, Zagreb, Croatia)
Oral presentation: "Untangling insect pathogenicity in plant-associated pseudomonads by a combination of comparative genomics and bioassays"

5th Swiss Microbial Ecology (SME) Meeting (4.-6. February 2013, Murten, Switzerland)
Poster: "How can plant-associated pseudomonads with antifungal activity become insect pathogens?"

2012

14th International Symposium on Microbial Ecology (ISME14) (19.-24. August 2012, Copenhagen, Denmark)
Poster: "How can plant-associated pseudomonads with antifungal activity become insect pathogens?"

CURRICULUM VITAE

Pascale Christina Flury
Born 7th March 1987
Citizen of Basel (BS) and Balsthal (SO), Switzerland
One daughter, born 2015

2012-16 Doctor of Science (PhD)
ETH Zurich
Plant Pathology Group: Institute of Integrative Biology
Doctoral Program: Science and Policy
Title of the thesis: Genomics, molecular mechanisms and ecology of interactions of plant-beneficial pseudomonads with pest insects
Advisors: Prof. Dr. Bruce A. McDonald and Dr. Monika Maurhofer (ETH Zürich) and Dr. Christoph Keel (University of Lausanne)

2009-11 Master of Science (M Sc) in Plant Science
University of Basel
Title of the thesis: The Role of *At*Peps in local and systemic resistance
Advisors: Prof. Dr. Thomas Boller, Dr. Dominik Klauser

2006-9 Bachelor of Science (B Sc) in Biology
University of Basel