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Title:

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

Running title:

Insects: vectors for plant-beneficial pseudomonads

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Conflict of Interest

The authors declare no conflict of interest.

Abstract

The discovery of insecticidal activity in root-colonizing pseudomonads, best-known for their plant-beneficial effects, raised fundamental questions about the ecological relevance of insects as alternative hosts for these bacteria. Since soil bacteria are limited in their inherent abilities of dispersal, insects as vectors might be welcome vehicles to overcome large distances. Here, we report on the transmission of the root-colonizing, plant-beneficial and insecticidal bacterium *Pseudomonas protegens* CHA0 from root to root by the cabbage root fly, *Delia radicum*. Following ingestion by root-feeding *D. radicum* larvae, CHA0 persisted inside the insect host throughout different life stages, a phenomenon that could be observed also in three other insect species. Next, CHA0 was successfully transmitted to the roots of a new plant host by emerging flies. Because *D. radicum* is a major root pest on various cabbage crops, we further assessed the biocontrol potential against this insect species. In summary, this study investigated for the first time the interaction of *P. protegens* CHA0 and related strains with an insect present in their natural habitat - the rhizosphere. Our results suggest that fluorescent pseudomonads can use insects as hosts and vectors, which implies a greater ecological versatility of these bacteria than previously thought.

Introduction

Every year worldwide crop production is facing major harvest losses due to plant pathogens and pest insects. Belowground attackers are especially difficult to tackle with chemical pesticides and adverse environmental effects of these products demand for alternative strategies such as the use of antagonistic organisms to control pest organisms, known as biological control. Root-colonizing bacteria of the *Pseudomonas fluorescens* group have been extensively studied for their beneficial effects on plants, e.g. the suppression of root diseases and the promotion of plant-growth (Haas and Défago, 2005). This research provides us on one hand with a profound knowledge on the interaction of these bacteria with the plant host and with antagonistic microbes in the soil (Vacheron *et al.*, 2013) and on the other hand already led to several commercial products (Berg, 2009).

More recent studies revealed that the phylogenetically distinct *Pseudomonas chlororaphis* subgroup with the two representative species *Pseudomonas chlororaphis* and *Pseudomonas protegens* in addition harbors features to colonize insects as an alternative habitat (Flury *et al.*, 2016; Kupferschmied *et al.*, 2013; Rangel *et al.*, 2016; Ruffner *et al.*, 2015). Strains of the *P. chlororaphis* subgroup were found to exhibit oral activity against larvae of Lepidoptera (Flury *et al.*, 2016; Rangel *et al.*, 2016; Ruffner *et al.*, 2013) as well as against *Drosophila melanogaster* (Olcott *et al.*, 2010) and a *P. chlororaphis* toxin was found to be active against the western corn rootworm *Diabrotica virgifera virgifera* (Schellenberger *et al.*, 2016). Several factors have been identified to contribute to insect pathogenicity: the Fit toxin, antimicrobial metabolites, secreted enzymes, lipopolysaccharide O antigen and the insecticidal protein IPD072Aa (Devi and Kothamasi, 2009; Flury *et al.*, 2016; Flury *et al.*, 2017; Jang *et al.*, 2013; Keel, 2016; Kupferschmied *et al.*, 2016; Loper *et al.*, 2016; Olcott *et al.*, 2010; Péchy-Tarr *et al.*,

2008; Ruffner *et al.*, 2013; Ruffner *et al.*, 2015; Schellenberger *et al.*, 2016). In-depth studies on the Fit toxin in the model strain *P. protegens* CHA0 revealed that the bacteria produce this insecticidal protein specifically in insects, but not on plant roots (Kupferschmied *et al.*, 2014; Péchy-Tarr *et al.*, 2013). Accordingly, the bacteria seem to sense their environment and regulate the production of specific compounds depending on the specific needs in the encountered habitat.

Although there is a growing body of evidence that insects represent an alternative host for *P. chlororaphis* subgroup bacteria, the ecology of their insect-associated lifestyle is still elusive. To date, oral insecticidal activity has been investigated only in model insects feeding on leaves (Flury *et al.*, 2016; Flury *et al.*, 2017; Kupferschmied *et al.*, 2014; Rangel *et al.*, 2016; Ruffner *et al.*, 2013). However, *P. protegens* CHA0 and related strains of the *P. chlororaphis* subgroup were isolated from roots and their interaction with root-feeding insects is therefore of much greater ecological relevance. It is still unknown whether these bacteria are also pathogenic to soil insects and therefore have a potential as biocontrol organisms of root pests. Furthermore, the discovery that several plant-beneficial pseudomonads exhibit specific adaptions to a life in insects (Keel, 2016; Kupferschmied *et al.*, 2013; Kupferschmied *et al.*, 2014; Kupferschmied *et al.*, 2016) raised the hypothesis that insects might represent attractive vectors to reach new plant hosts. Rhizobacteria are limited in their inherent dispersal abilities and may largely depend on passive transport, such as water flows, to overcome large distances. Alternatively, dispersal by means of a vector is a plausible manner of attaining new habitats. Insect-mediated dispersal has been described for several plant-pathogenic bacteria (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), but mainly when insect vectors were feeding on L11-infested foliage. *P. protegens* CHA0, in contrast, was not found to move to above ground plant parts (Iavicoli *et al.*, 2003; Maurhofer *et al.*, 1998). For the dispersal of a rhizobacterium, which is restricted to below-ground plant parts, an insect with a root-feeding larval and an above-ground flying adult stage would represent a suitable vector. A prerequisite for this kind of dispersal is persistence of the bacteria in the insect host and transstadial transmission from larva over pupa to the adult stage.

This study investigates for the first time the interaction of *P. protegens* CHA0 with a root-feeding pest insect, the cabbage fly *Delia radicum*. Their larvae feed on brassicaceous plants, pupate in the soil and emerging adults fly to a new host plant to deposit eggs. While all *P. chlororaphis* subgroup strains tested so far are to a high degree lethal to many lepidopteran insect species (Flury *et al.*, 2016; Rangel *et al.*, 2016), we found in the present study survival of *D. radicum* larvae to be affected by certain strains of the *P. chlororaphis* subgroup, but not by *P. protegens* CHA0. Nevertheless, CHA0 was able to persist in *D. radicum* throughout different life-stages and adult flies emerging from larvae that fed on CHA0 colonized roots transmitted the bacteria to the roots of new host plants. This provides the first direct evidence for the possibility of insect-mediated dispersal of *P. protegens* CHA0. Persistence of CHA0 throughout different life-stages seems to be a rather general phenomenon as it was observed in three further insect species, tested in this study. Overall our data indicate that indeed soil insects might be both, relevant alternative hosts and vectors for certain plant-beneficial rhizobacteria.

Materials and Methods

Bacterial cultures

The bacteria used in this study are listed in Table 1. Strains with a constitutively expressed GFP tag were generated by means of the Tn7 delivery vectors pBK-miniTn*7-gfp1* or pBKminiTn*7-gfp2* (Kupferschmied *et al.*, 2014). In the results and discussion sections we always use wild-type names of strains. Whether GFP-tagged variants were used is indicated in the materials and methods and in the figure legends. Generally, GFP-tagged strains did not differ in their activity from the respective wild-type strains. Bacteria were cultured in lysogeny broth (LB), supplemented with either kanamycin (25 µg/ml) or gentamicin (10 µg/ml) for GFP expressing strains, overnight at 24°C and 180 rpm. For the cauliflower experiments with *D. radicum* 200 µl of LB cultures were used to inoculate King's B (KB) agar plates (King *et al.*, 1954) supplemented with gentamicin (10 µg ml^{-1}). After one day, bacterial cultures were scrapped off the plates, suspended, washed twice in sterile ddH_2O, and OD_{600} was adjusted to the desired concentration. In all other experiments, LB cultures were washed twice (once for radish experiments) in sterile 0.9% NaCl solution or water before adding cell suspensions adjusted to the desired concentration to diets or roots/radish, respectively.

Radish experiment with *D. radicum*

D. radicum was reared as described by Razinger *et al.* (2014), but larvae were fed on turnip cabbage instead of rutabaga. Greens of organically grown radishes (Migros, Switzerland) were cut off about 0.5 cm above the bulbs. Those were then washed with tap water and 70% ethanol, dried with household paper and submerged for 10 min in a bacterial suspension of OD_{600} of 0.47 or ddH_2O as a control. Then the radishes were buried in pots (345x276x80 mm)

(Bachmann Plantec AG, Switzerland) filled with sterile quartz sand. Eight eggs of *D. radicum* were deposited on top of the sand and the pots covered with aluminium foil were incubated in a climate chamber (16-h day, 20°C, 210 μmol m^{-2} s^{-1}; 8-h night cycle, 18°C) for four weeks. In experiment two only six eggs were added per radish, because radish bulbs were smaller. Developing pupae were harvested by washing the sand over a sieve. All pupae emerging from one pot were photographed together and pupal size was measured by means of an ImageJ macro. Two weeks later, flies emerging from pupae were quantified. Flies of the CHA0-*gfp2* and control treatments were checked for presence of CHA0 by incubating entire flies each in 1 ml LB supplemented with chloramphenicol (13 μg ml^{-1}), cycloheximide (100 μg ml^{-1}) and gentamicin (10 μg ml^{-1}) for two days. To verify the identity of the growing bacteria, they were checked for GFP expression under a Leica DM2500 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). The experiment was conducted twice.

Cauliflower experiment with *D. radicum*

Cauliflower plants (four trays each containing twelve pots, one plant per pot) were grown with and without *P. protegens* CHA0-*gfp2* for three weeks as detailed in the Supplementary Methods. Then, five freshly hatched *D. radicum* larvae were added to each plant. Four weeks later, plant shoots were weighed and root systems were washed on a sieve to collect pupae as well as non-pupated larvae. The latter were directly extracted for bacteria monitoring. Bacterial root colonization was assessed as described in Supplementary Methods. Pupal size was measured as described for the radish experiments. Ten to twelve pupae per treatment were extracted to assess colonization by inoculant bacteria, three to four pupae were transferred to each of the transmission microcosms and the remaining pupae (44-56 per treatment) were observed to

200 determine hatching rates. The transmission microcosms were designed to test whether flies are
201 transmitting CHA0 to the roots of a new host plant and are described in detail in the
202 Supplementary Methods. Briefly, each transmission microcosm consisted of four rapeseed plants
203 grown axenically on a sand-vermiculite substrate in closed plastic beakers. Three to four pupae
204 that had emerged from control or *P. protegens* CHA0-*gfp2* treatments were added separated from
205 plants and substrate to each transmission microcosm. Nine days after flies had started to emerge
206 and fly around inside the microcosms, roots of rapeseed plants were checked for colonization by
207 *P. protegens* CHA0-*gfp2* as described for cauliflower plants (Supplementary Methods). Roots of
208 plants grown in the same beaker were pooled for analysis.
209
210 **Survival and colonization experiments with *Plutella xylostella* and *Pieris brassicae***
211 The experiments with *P. xylostella* were conducted as detailed in Flury *et al.* (2017) and
212 are briefly described in the Supplementary Methods. *P. brassicae* larvae were reared at 25°C,
213 60% relative humidity and a 16-h day, 8-h night cycle and fed with Brussels sprouts variety
214 Topline F1. During the experiments larvae were kept individually in Petri dishes lined with a
215 moisturized filter paper and were fed with a pellet of artificial diet (David and Gardiner, 1965)
216 inoculated with 10 µl of suspension of *P. protegens* CHA0-*gfp2* or CHA0 cells at an OD_{600} of 20
217 or amended with sterile 0.9% NaCl solution (control). Larvae that did not consume the entire diet
218 pellet were excluded from the experiment. After 24 h, larvae were transferred in groups of six
219 into 720 ml Pint-sized BugDorms (BugDorm, Taiwan) and fed with cabbage until pupation. 24 -
220 32 larvae per treatment were used for monitoring mortality. Larvae and pupae were considered
221 dead when they did not react to poking. Further individuals (alive, crippled and dead) of each

222 developmental stage were extracted as described below. The experiment was conducted three
223 times.
224
225 **Assessment of bacterial colonization rates**
226 To assess bacterial colonization rates, insects were surface sterilized (20 s 70% ethanol,
227 20 s sterile ddH_2O for *P. xylostella* and *P. brassicae*; 20 s 0.05% SDS, 20 s 70% ethanol, 20 s
228 sterile ddH_2O for *D. radicum*) and then homogenized in sterile 0.9% NaCl solution with a
229 Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). The resulting suspensions were
230 serially diluted and plated onto KB agar plates supplemented with chloramphenicol (13 μg ml^{-1}),
231 cycloheximide (100 μg ml^{-1}) and gentamicin (10 μg ml^{-1}). For *D. radicum* plates were
232 additionally supplemented with ampicillin (40 μg ml^{-1}). GFP-expression of growing colonies was
233 verified under the microscope (ex: 480/BP 40 nm, em: 527/BP 30 nm).
234
235 **Microscopy**
236 Microscopic investigations and sample preparation of *P. xylostella* larvae are described in
237 Supplementary Methods.
238
239 **Statistics**
240 Data analysis was performed in RStudio version 0.98.1017 (http://www.rstudio.com)
241 using R version 3.1.2. Data were tested for normal distribution (Shapiro-Wilk test) and
242 homogeneity of variance and according to the results a Student's t test or a Mann-Whitney *U* test
243 (cauliflower experiments) or a Kruskal Wallis (radish experiment) was performed. For *Pieris*
244 experiments, the Log-Rank test of the Survival package of R and a Chi-square test were used to

compare survival curves and numbers of dead and crippled individuals between treatments, respectively.

Results

Minor effects of *P. chlororaphis* subgroup bacteria against the root pest *D. radicum*

To investigate for the first time how bacteria of the *P. chlororaphis* subgroup, known for their insecticidal activity against various leaf-feeding insects (Flury *et al.*, 2017; Rangel *et al.*, 2016; Ruffner *et al.*, 2013), interact with an insect living in their natural habitat, the rhizosphere, we tested the oral activity of these bacteria to the cabbage fly *D. radicum*, an important root pest on brassicaceous crops.

In experiments with bacteria treated radish, aiming at comparing different bacterial strains, *P. chlororaphis* PCL1391 caused a significant reduction of the pupation rate and the pupal size compared to the control (Figure 1A, C). Moreover, in this experiment a reduction in pupal size was also observed for the strains *P. protegens* CHA0 and *Pseudomonas* sp. CMR12a. In experiment two only *Pseudomonas* sp. CMR12a caused a significant reduction in pupation rate and pupal size (Figure 1B, D).

In the cauliflower experiment, with larvae feeding on colonized roots, *P. protegens* CHA0 developed on average population sizes of 6.51 ± 0.59 and 5.92 ± 0.56 $\log_{10}$ cfu per g of root fresh-weight in the two experiments. In both experiments, no significant differences in pupation rate, pupal size and in the number of flies emerging from pupae could be detected between control and CHA0 treatments (Figure 2A, Supplementary Figure S1, Supplementary Figure S2A, B). Moreover, shoot weights from plants inoculated with CHA0 did not significantly differ from those of control plants (Supplementary Figure S2C, D).

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

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

In a previous study, we reported that certain strains of the *P. fluorescens* group that are not causing fatal infections are still able to persist inside larvae of the cotton leafworm *Spodoptera littoralis* (Flury *et al.*, 2016). We were wondering whether, although no effect on survival of *D. radicum* larvae was observed in the present study, *P. protegens* CHA0 is able to colonize the larvae and to persist inside the insect throughout different life stages. Extraction of *Delia* larvae and pupae from the two cauliflower experiments showed that they indeed were colonized by CHA0 (Figure 2B, Figure 3) at average levels of 2.7 $\log_{10}$ and 3.8 $\log_{10}$ cfu per insect. No CHA0 was detected in insects from the control treatment. In contrast to results on larvae and pupae, our method of mixing the flies and plating serial dilutions generally revealed no *P. protegens* CHA0 associated with adult flies whether these were surface-sterilized or not. However, a few flies that emerged from CHA0 treated roots showed developmental defects, most obviously malformations of the wings (Figure 2C). Three crippled flies were extracted and remarkably two of them were colonized by CHA0 (Figure 2B, Figure 3). Since healthy looking flies from the cauliflower experiments were able to transmit CHA0 to a new host plant (see results below), they must have carried the bacteria although we did not detect them with our extraction method. Therefore, we assessed larger amounts of *Delia* flies for presence of CHA0 by a second method. Entire flies emerging from control and CHA0 treatments of radish experiments were simply put in selective liquid medium without prior surface disinfestation and the medium was then checked for growth of CHA0. This qualitative approach revealed that in one experiment 76% (n=21) and in the other experiment 53% (n=30) of healthy flies were carrying CHA0. No CHA0 was detected on flies from the control treatment.

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

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

To assess, whether *Delia* flies which had been exposed to *P. protegens* CHA0 at the larval stage are able to transmit CHA0 to a new host plant, we elaborated a specific test system. Pupae that emerged from control and CHA0 treatments in the cauliflower experiments were transferred into closed plastic beakers (three to four pupae per beaker) containing rapeseed plantlets grown axenically on a sand-vermiculite substrate. To avoid transmission of bacteria by the pupae, those were kept in containments preventing direct contact with plants or substrate. Emerging flies were flying around in the transmission microcosms and in several of them they also laid eggs. Nine days after first flies started to emerge, root systems were assessed for bacterial colonization. The roots of twelve out of thirteen (experiment one) and of eight out of nine (experiment two) transmission microcosms, which had been exposed to flies emerging from the CHA0 treatment, were indeed colonized by *P. protegens* CHA0 (Figure 2D, Supplementary Figure S3). In both experiments, no CHA0 could be detected on roots from transmission microcosms that had been exposed to control flies (Figure 2D, Supplementary Figure S3). In microcosms with successful CHA0 transmission, average colonization rates in experiment one and two were 5.0 $\log_{10}$ and 4.3 $\log_{10}$ cfu per g of roots, respectively. Hence, *Delia* flies were able to transmit *P. protegens* CHA0 to a new host plant (Figure 3).

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 differing in their susceptibility to a CHA0 infection: the leaf-feeders diamondback moth *Plutella xylostella* and large white butterfly *Pieris brassicae* and the root-feeder black vine weevil *Otiorhynchus sulcatus*.

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

Although several insect pathogenicity factors of *P. protegens* CHA0 have been identified (Keel, 2016), very little is known about the infection process inside the insect and the damage caused by CHA0. To be able to study in the same insect the localization of CHA0 and histological changes of insect tissues we established a microscopy method on thin sections of *P. xylostella* larvae (Supplementary Methods). Larvae fed on CHA0 containing

diet were fixed and consecutive sections were either stained with anti-GFP antibodies or Heidenhain's iron hematoxylin to visualize the bacteria or the insect tissue, respectively. Larvae coming from two independent experiments were analyzed and representative pictures are shown in Figure 4 D-K. They give a first insight into colonization of *P. xylostella* larvae by *P. protegens* CHA0. In most larval samples from early infection time-points CHA0 could not be detected, but in two cases it was found in the midgut lumen (Figure 4D, E). In contrast, at later stages of infection CHA0 was often found in the hemolymph and the fat body cells while no excessive destruction of the midgut epithelium and no bacteria in the gut could be observed (Figure 4F, G, I). Finally, moribund larvae were always full of CHA0 all over the hemocoel and the gut and organs were not distinctively recognizable anymore (Figure 4J).

In oral infections of *P. brassicae* larvae with *P. protegens* CHA0, survival was found to be dependent on the larval stage. When 1st, 2nd and 3rd instar larvae were fed with high dosages of CHA0, approximately 70% to 95.8% of the larvae died within six days (Supplementary Figures S4A, S4B, S4C). In contrast, 4th instar larvae fed with the same number of CHA0 cells showed survival rates of over 95% in most of the experiments (Figure 5B, Supplementary Figures S4D, S5) and therefore this instar was used to assess persistence of CHA0 throughout different developmental stages (Figure 5). Still some CHA0-infected 4th instar larvae were unable to form intact pupae (Figure 5A6) and some pupae with normal appearance became melanized and died (Figure 5A7). The number of dead individuals (larvae plus pupae) was significantly higher in the CHA0 treatment compared to the control (Chi-Square, p=3.95e-05) (Figure 5C). Moreover, 15.5% of butterflies from the CHA0 treatment emerged with morphological defects, i.e. strongly deformed wings (Figure 5A8, 5C), which was again significantly higher than in the control (4.4%) (Chi-Square, p=0.018). Extraction of larvae, pupae and adults revealed presence of CHA0 in nearly all living larvae

at levels of around 4 $\log_{10}$ cfu per insect and in dead larvae even at levels as high as 9 $\log_{10}$ cfu per insect while in living pupae and adults with healthy appearance, CHA0 was only found exceptionally (Figure 5D). In individuals with abnormal phenotypes, CHA0 was often detected, which indicates that in certain cases it can survive in *P. brassicae* until the pupal and the adult stages (Figure 5D).

Similar to the results with *Delia* larvae feeding on cauliflower roots, CHA0 did not affect the survival of larvae of the root pest *O. sulcatus* feeding on strawberry roots (Supplementary Figure S6). However, CHA0 was qualitatively detected (Supplementary Methods) in two thirds (experiment A and B) of the pupae and in two thirds or all (experiment A and B, respectively) of *O. sulcatus* adults emerging from the CHA0 fed larvae.

Discussion

The here presented experiments provide first evidence that the root-colonizing *P. protegens* CHA0 can be dispersed by *D. radicum* to a new host plant and thus insects might not only serve as additional hosts for *P. chlororaphis* subgroup bacteria, but also as vectors. A summary of a potential transmission cycle is depicted in Figure 3. *P. protegens* becomes internalized by root feeding *Delia* larvae, persists until the pupal stage and emerging flies can transmit the bacterium to the roots of a new host plant (Figure 3). This could, for instance, occur when female flies lay eggs next to plant stems, thereby delivering bacteria directly into a new soil habitat. Alternatively, *P. protegens* might be transmitted to plant shoots and washed into the soil by rainfall. In the following, the bacterium colonizes the roots of the new host plant and can again colonize larvae that hatched from deposited eggs (Figure 3). As vectors, insects would allow the bacteria to overcome large distances and to conquer new root habitats, which might considerably influence the spread of root-colonizing fluorescent pseudomonads. Insect-mediated dispersal is also known for several plant-pathogenic bacteria,

but most of them do not exhibit insecticidal activity (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 plant-pathogens are also insect-pathogenic. *Pseudomonas syringae* B728a can cause high mortality rates in the pea aphid and at the same time, it is excreted with honeydew and can thereby be dispersed by moving aphids (Stavrinides *et al.*, 2009). Our results indicate that the susceptibility of the root pest *D. radicum* to *P. chlororaphis* subgroup bacteria depends on the bacterial strain, but is generally smaller than the susceptibility of the lepidopteran leaf-feeders tested here and in earlier studies (Flury *et al.*, 2016; Rangel *et al.*, 2016; Ruffner *et al.*, 2013). However, those 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 radishes or 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). Nevertheless, for certain of the tested strains reduced larval survival and pupal size were observed indicating that they can be pathogenic to *D. radicum*.

Morphological defects in adult *P. brassicae* and *D. radicum* co-occurring with the presence of *P. protegens* CHA0 are an indication for negative effects of CHA0 on insect development. Similar observations are reported for *D. melanogaster* larvae infected with the related strain *P. protegens* Pf-5 (Loper *et al.*, 2016; Olcott *et al.*, 2010) and for leaffolder moths (*Cnaphalocrocis medinalis*) fed with rice leaves treated with a mix of *P. fluorescens* strains (Saravanakumar *et al.*, 2007). We hypothesize that *P. chlororaphis* subgroup bacteria are opportunistic pathogens for *D. radicum* and able to infect weakened individuals. For biocontrol purposes, bacterial effects might be increased by exposing *D. radicum* to

421 additional stress, e.g. by combining bacteria with organisms that could facilitate the access to
422 the hemocoel, such as entomopathogenic fungi or nematodes.

423 A prerequisite for insect-mediated dispersal of *P. protegens* CHA0 is the persistence
424 inside the insect. Transstadial transmission (from larva to adult) of bacteria that do not exhibit
425 an intracellular lifestyle, which is common for endosymbionts (Engel and Moran, 2013;
426 Kikuchi, 2009), is reported for several insect species (Chavshin *et al.*, 2015; Greenberg and
427 Klowden, 1972; Moll *et al.*, 2001; Radvan, 1960). However, in other cases bacteria are lost
428 during pupal stage, before adult emergence (Greenberg and Klowden, 1972; Leach, 1934;
429 Moll *et al.*, 2001; Radvan, 1960). In *D. radicum* and *O. sulcatus*, *P. protegens* CHA0 was
430 transstadially transmitted, without affecting insect survival. Our qualitative approach, where
431 entire flies were incubated in selective medium, detected CHA0 associated with adult *D.*
432 *radicum* in contrast to our extraction method. Moreover, our transfer experiments showed
433 that the bacterium was transmitted by *Delia* flies in about 90% of the cases. These results
434 indicate that emerging flies regularly carry CHA0 but in very low numbers (detection limit of
435 the extraction method: 10-100 cells). Due to repeated molting and metamorphosis, the insect
436 represents an unstable habitat for microbes, though bacteria might still persist in specialized
437 crypts or paunches present in the guts of many insect species (Engel and Moran, 2013).
438 Persistence of CHA0 in insects of different orders each of which harbors specific anatomical
439 and developmental features might rely on different strategies.

440 In contrast to *D. radicum* and *O. sulcatus*, larvae of *P. xylostella* and *P. brassicae* are
441 susceptible to CHA0 and become highly colonized. In *P. xylostella* CHA0 generally persists
442 throughout all developmental stages, but the insects seem to succumb to the infection sooner
443 or later. In contrast, a considerable fraction of 4th instar *P. brassicae* larvae was able to
444 eradicate CHA0 before or during pupation. However, those *Pieris* larvae, in which CHA0
445 was able to persist, seemed to succumb to the infection during pupation or exhibited major

446 developmental defects. During a lethal infection, *P. protegens* CHA0 multiplies to very high
447 numbers as shown in Figure 4 and reported earlier (Flury *et al.*, 2016; Kupferschmied *et al.*,
448 2013; Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013). Extraction of entire larvae does not allow
449 any conclusion on the localization of the bacteria. The here presented microscopical method
450 enabled the visualization of *P. protegens* CHA0 during the insect infection and, in parallel,
451 the observation of histopathological changes in *P. xylostella* larvae. First observations
452 indicate that *P. protegens* CHA0 does not colonize the gut to very high numbers and does not
453 cause complete rupture of the midgut epithelium. Therefore, we hypothesize that *P.*
454 *protegens* CHA0 colonizes a restricted area of the gut, where it is able to enter the hemocoel.
455 Once in the hemocoel the bacteria multiply exponentially causing a fatal septicemia. The use
456 of the insect body as a mass replication vessel is supported by the pictures of moribund larvae
457 as well as by the very high bacteria counts in dead individuals of *P. xylostella* and *P.*
458 *brassicae*. The pictures presented here give only a first insight into the colonization and
459 infection process. How and where exactly the bacterium overcomes the gut barrier in order to
460 invade the hemolymph remains to be discovered and requires more in-depth microscopical
461 studies.

462 This study investigated for the first time different aspects of how *P. chlororaphis*
463 subgroup bacteria, known for their insecticidal activity against leaf-feeding insects, interact
464 with a root-feeding insect present in their natural habitat, the rhizosphere. While the reduction
465 of larval survival by certain strains gives hope for potential applications as biocontrol
466 organisms of root pests, such as *D. radicum*, the discovery of persistence of *P. protegens*
467 CHA0 in insects throughout different developmental stages and its dispersal to a new host
468 plant adds novel and intriguing aspects to the ecology of fluorescent pseudomonads. These
469 bacteria seem to be much more versatile than previously thought and we are still far from
470 fully understanding their ecology. It is for instance still unknown how often and in which

relation, e.g. whether as commensals or pathogens, plant-beneficial pseudomonads are associated with natural insect populations. It also remains subject to future research to discover additional habitats these bacteria might have conquered and to elucidate how they manage to switch between very different, e.g. root- and insect-associated, life styles.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

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

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

683 Colonization levels of CHA0-*gfp2* on roots of rapeseed plants. Plants were exposed for nine
684 days to flies, which as larvae had fed on roots of control or CHA0-*gfp2* treated cauliflower
685 plants, i.e. the previous plant host. In the CHA0 treatment, in twelve out of thirteen systems
686 roots became colonized with *P. protegens* CHA0-*gfp2*. A repetition of the experiment is
687 depicted in Supplementary Figure S3. **(B, D)** Numbers above boxes indicate sample size.
688
689 **Figure 3. *Pseudomonas protegens* CHA0, taken up by root-feeding *Delia radicum* larvae,**
690 **persists in the insect throughout different life stages and is dispersed to new host plants.**
691 Roots of cauliflower plants were inoculated with *P. protegens* CHA0 **(1)** and freshly hatched
692 larvae of *D. radicum* were added to feed on the colonized roots **(2)**. CHA0 was ingested by
693 the larvae **(3)** and found to persist inside the insect also in the pupal **(4)** and adult stage **(5, 6)**
694 (Figure 2B). A few flies emerging from pupae exhibited morphological defects **(5),** which
695 affected mainly the wings (Figure 2C). In nature, crippled flies are not able to reproduce,
696 which will cause a decline of the insect population. However, healthy flies **(6)** will mate and
697 females will search for a new host plant where they deposit their eggs in the immediate
698 vicinity of the stem **(7)**. Flies are able to transmit the bacterium to the roots of a new host
699 plant resulting in high bacterial colonization **(8)** (Figure 2D).
700
701 **Figure 4. Infection of *Plutella xylostella* by *Pseudomonas protegens* CHA0. (A-C)** One-
702 week-old *P. xylostella* larvae (n=32-64) were exposed to artificial diet inoculated with a low
703 dosage (low, 10 µl of OD_{600} = 0.01) or a high dosage (high, 10 µl of OD_{600} = 0.1) of *P.*
704 *protegens* CHA0-*gfp2* or amended with sterile 0.9% NaCl solution (control). **(A)** Fraction of
705 *P. xylostella* larvae dying at larval or pupal stages or emerging as adults. Three repetitions of
706 the experiment are depicted. **(B)** Colonization of *P. xylostella* by *P. protegens* CHA0-*gfp2*
707 (high dosage). Data are pooled from experiments 2 and 3. **(C)** Colonization of *P. xylostella* by

P. protegens CHA0-*gfp2* (low dosage). Data are pooled from all three experiments. **(B, C)** Numbers above boxes indicate sample size. No *P. protegens* CHA0-*gfp2* was detected in control insects. **(D - K)** Tracking *P. protegens* CHA0 in *P. xylostella* larvae upon oral uptake using microscopy on serial sections of fixed larvae. Larvae were infected with *P. protegens* CHA1176, a GFP expressing variant of *P. protegens* CHA0. **(D-G, J)** Sections of these larvae were stained with anti-GFP. The use of anti-GFP antibodies was necessary, because of fixation of larvae with Duboscq-Brazil's alcoholic Bouin's destroys intrinsic GFP fluorescence. **(H, I)** Sections stained with Heidenhain's iron hematoxylin. **(D, E)** CHA0 in the gut, but not in the hemolymph. **(E)** is a magnification of **(D)**. **(F-I)** CHA0 in the hemolymph and in fat body cells, but not in the gut. **(H)** Control larva fed on bacteria free diet. **(J)** Moribund larva completely colonized by CHA0**. (K)** Consecutive section of **(J)** stained without adding anti-GFP antibody. B, bacteria; F, fat body; GL, gut lumen; H, hemocoel; ME, midgut epithelium.

Figure 5. Infection of *Pieris brassicae* by *Pseudomonas protegens* CHA0. (A-D) Fourth instar *P. brassicae* larvae were fed on artificial diet inoculated with 10 µl of a *P. protegens* CHA0-*gfp2* bacterial suspension (OD_{600}= 20) (CHA0) or amended with sterile 0.9% NaCl solution (control). **(A)** Phenotypical differences between insect stages developed from control (1-4) and CHA0 (5-8) treated larvae. Healthy larvae (1, 2), pupa (3) and butterfly (4); dead larva (5) and pupa (7); pupa (6) and butterfly (8) with morphological defects. **(B)** Impact of CHA0 on development and mortality of *P. brassicae* over time. Two repetitions of the experiment are depicted in Supplementary Figure S5. **(C)** Fate of larvae fed with control or CHA0 treated diet. Data are pooled from three independent experiments. Numbers of dead and crippled individuals differed significantly between the control and CHA0 treatments according to a Chi-Square test (p-value < 0.05). **(D)** Colonization of *P. brassicae* by *P.*

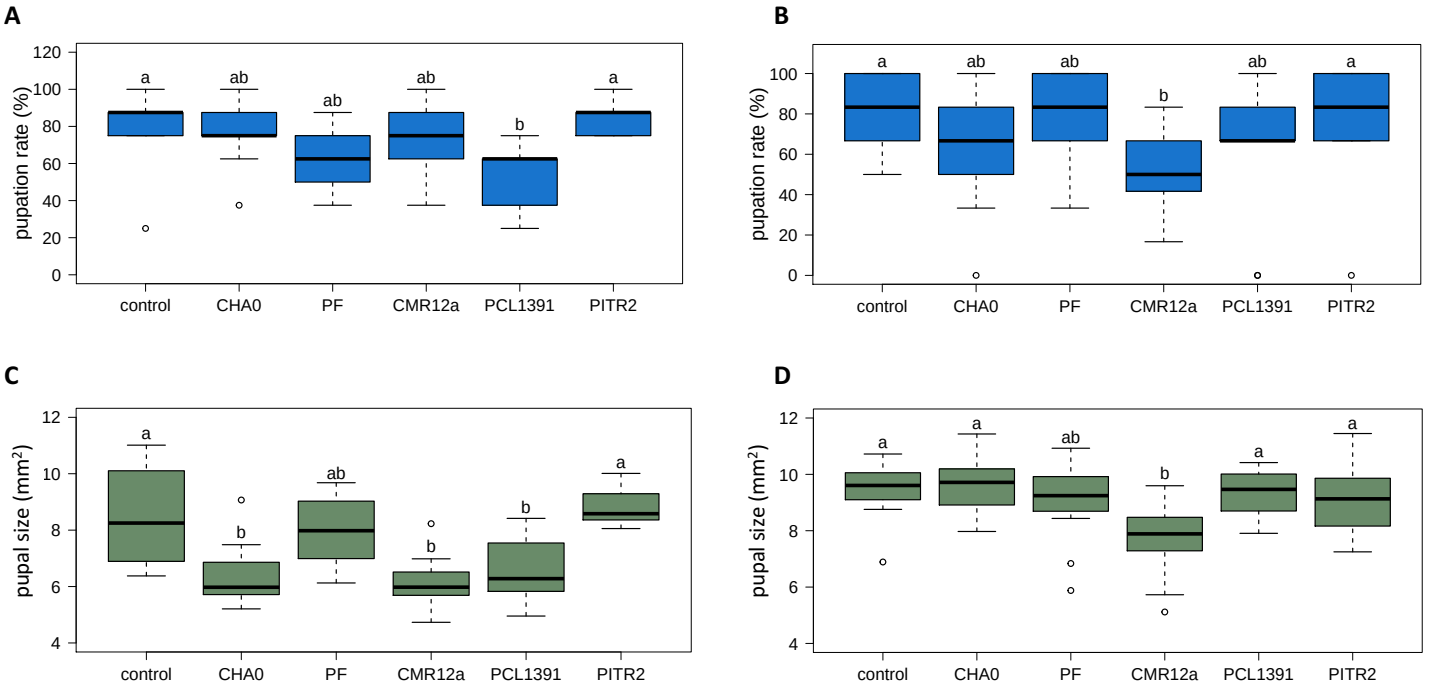
protegens CHA0-*gfp2*. Data are pooled from three independent experiments. Numbers above boxes indicate sample size. No *P. protegens* CHA0-*gfp2* was detected in control insects. Alive crippled pupae or butterflies, living individuals with abnormal phenotypes; dead crippled pupae, individuals that failed to form intact pupae and died; dead crippled butterflies, butterflies with morphological defects that died during emergence.

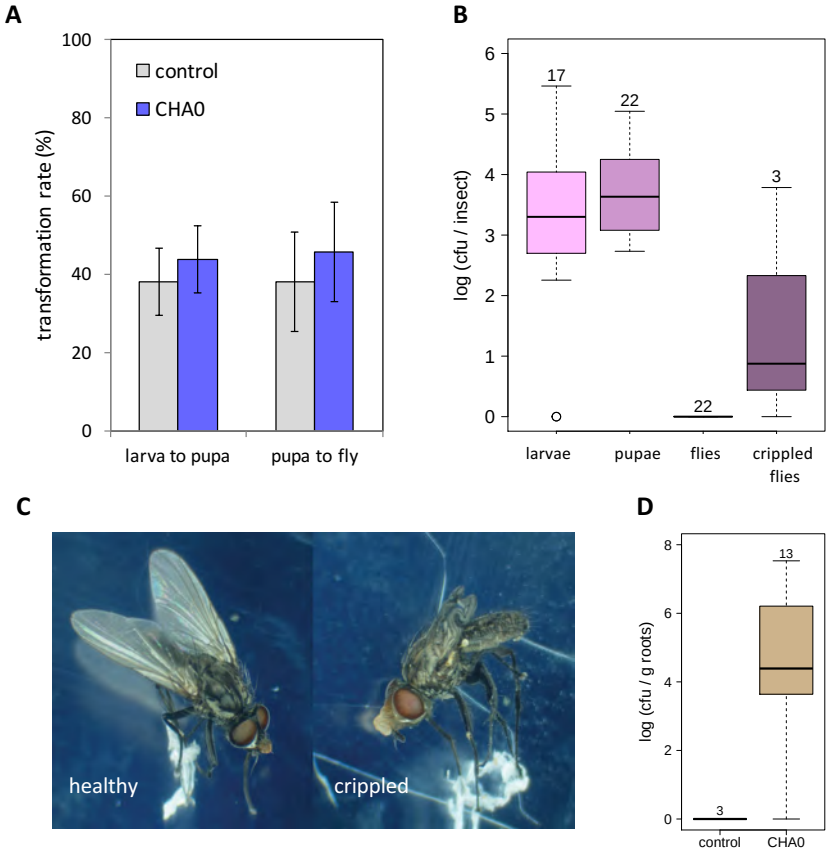
758

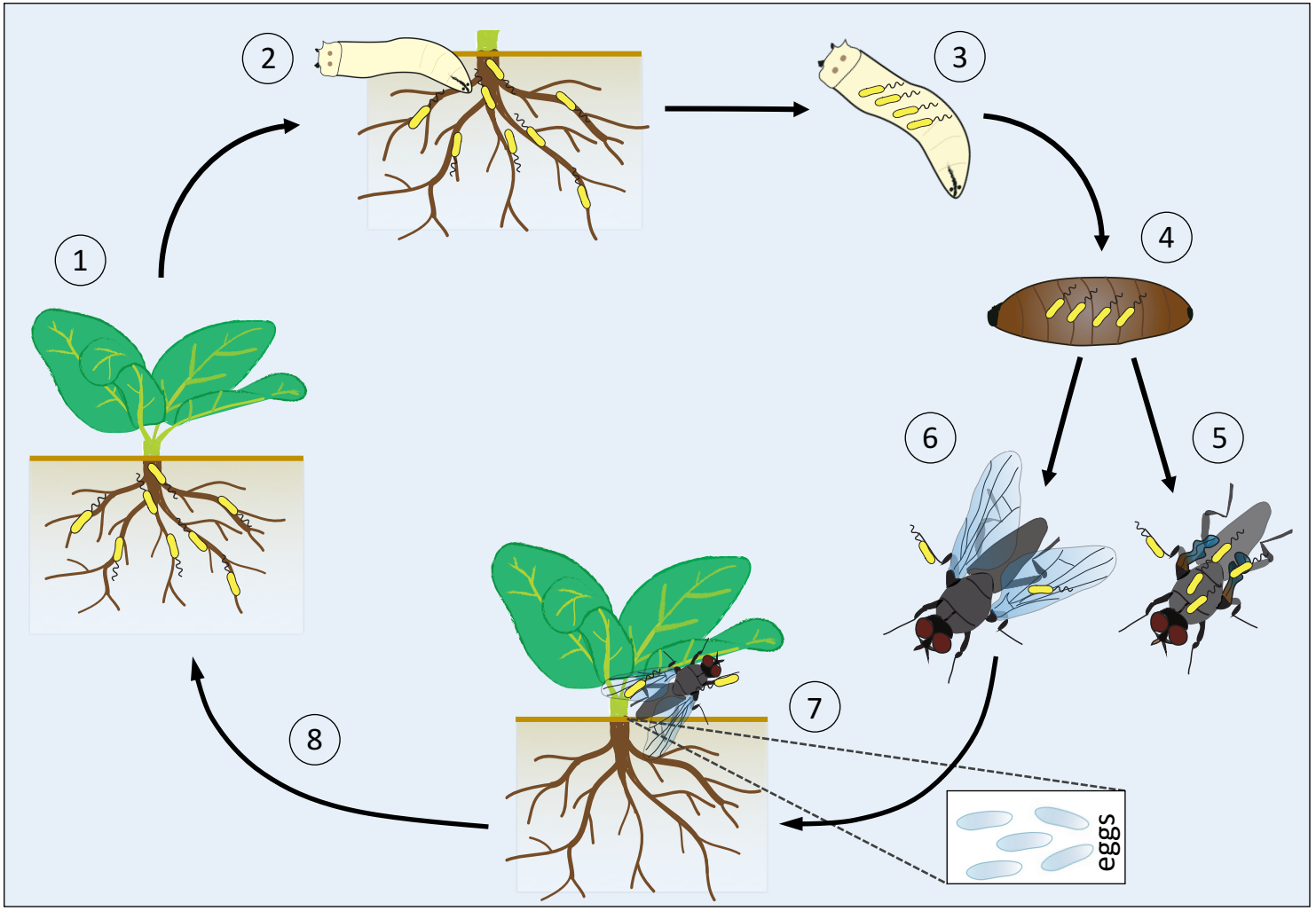
Table 1. Bacterial strains used in this study

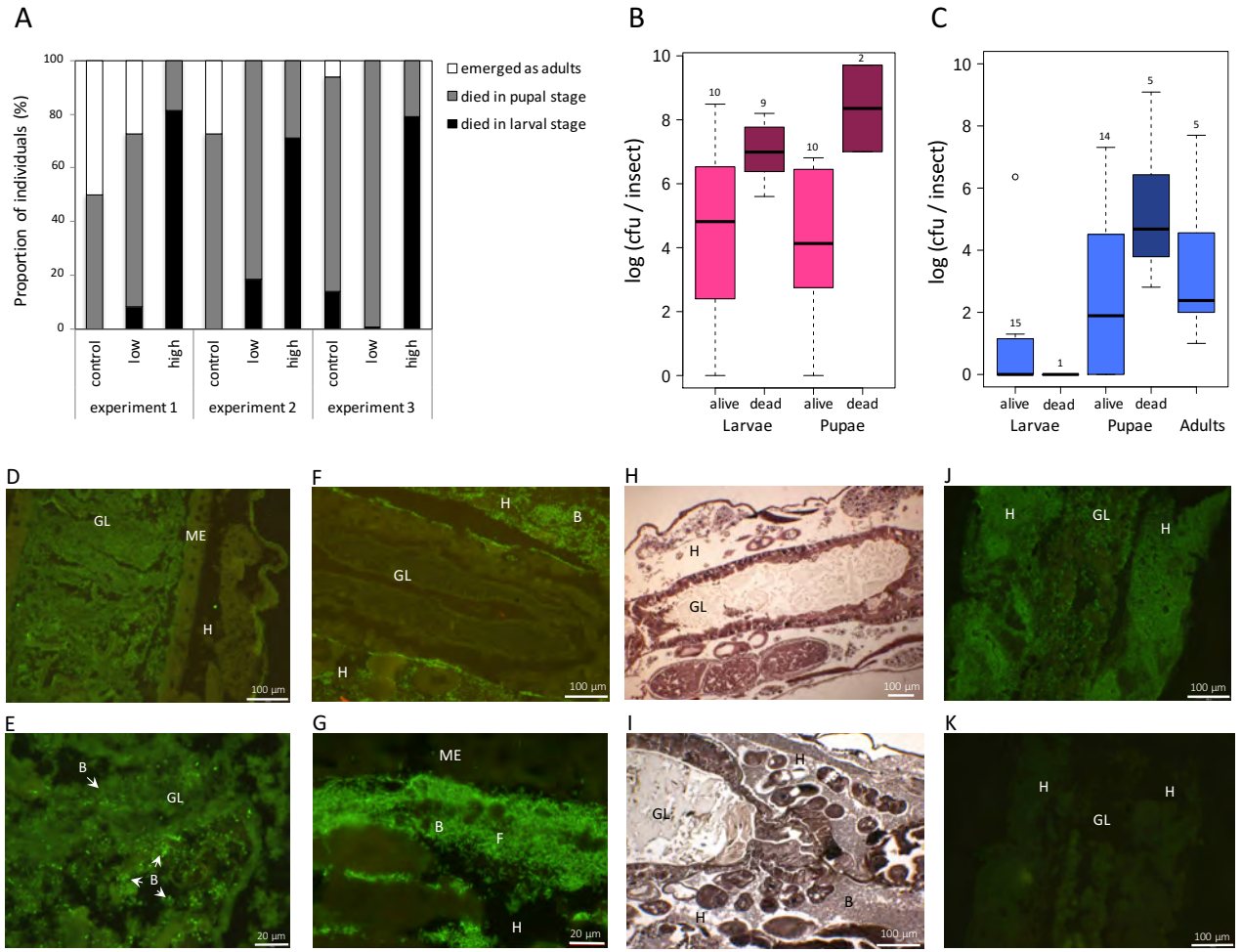
Strain	Genotype, phenotype or relevant characteristics	Insecticidal activity	Reference or source
Pseudomonas protegens CHA0	Wild type, isolated from tobacco roots	Yes	Jousset *et al.* (2014); Stutz *et al.* (1986)
Pseudomonas protegens CHA0-*gfp2*	CHA0::*att*Tn*7*-*gfp2;* Gm^r	Yes	Péchy-Tarr *et al.* (2013)
Pseudomonas protegens CHA1176	CHA0::*att*Tn*7*-*gfp2 fitD-mcherry;* Gm^r	Yes	Péchy-Tarr *et al.* (2013)
Pseudomonas protegens PF-*gfp2*	PF::*att*Tn*7*-*gfp2;* Gm^r	Yes	This study, for wild type PF see Levy *et al.* (1992)
Pseudomonas sp. CMR12a-*gfp1*	CMR12a::*att*Tn*7*-*gfp1;* Km^r	Yes	This study, for wild type CMR12a see Perneel *et al.* (2007)
Pseudomonas chlororaphis PCL1391-*gfp2*	PCL1391::*att*Tn*7*-*gfp2;* Gm^r	Yes	This study, for wild type PCL1391 see Chin-A-Woeng *et al.* (1998)
Pseudomonas thivervalensis PITR2-*gfp2*	PITR2::*att*Tn*7*-*gfp2;* Gm^r	No	This study, for wild type PITR2 see (Keel *et al.*, 1996)

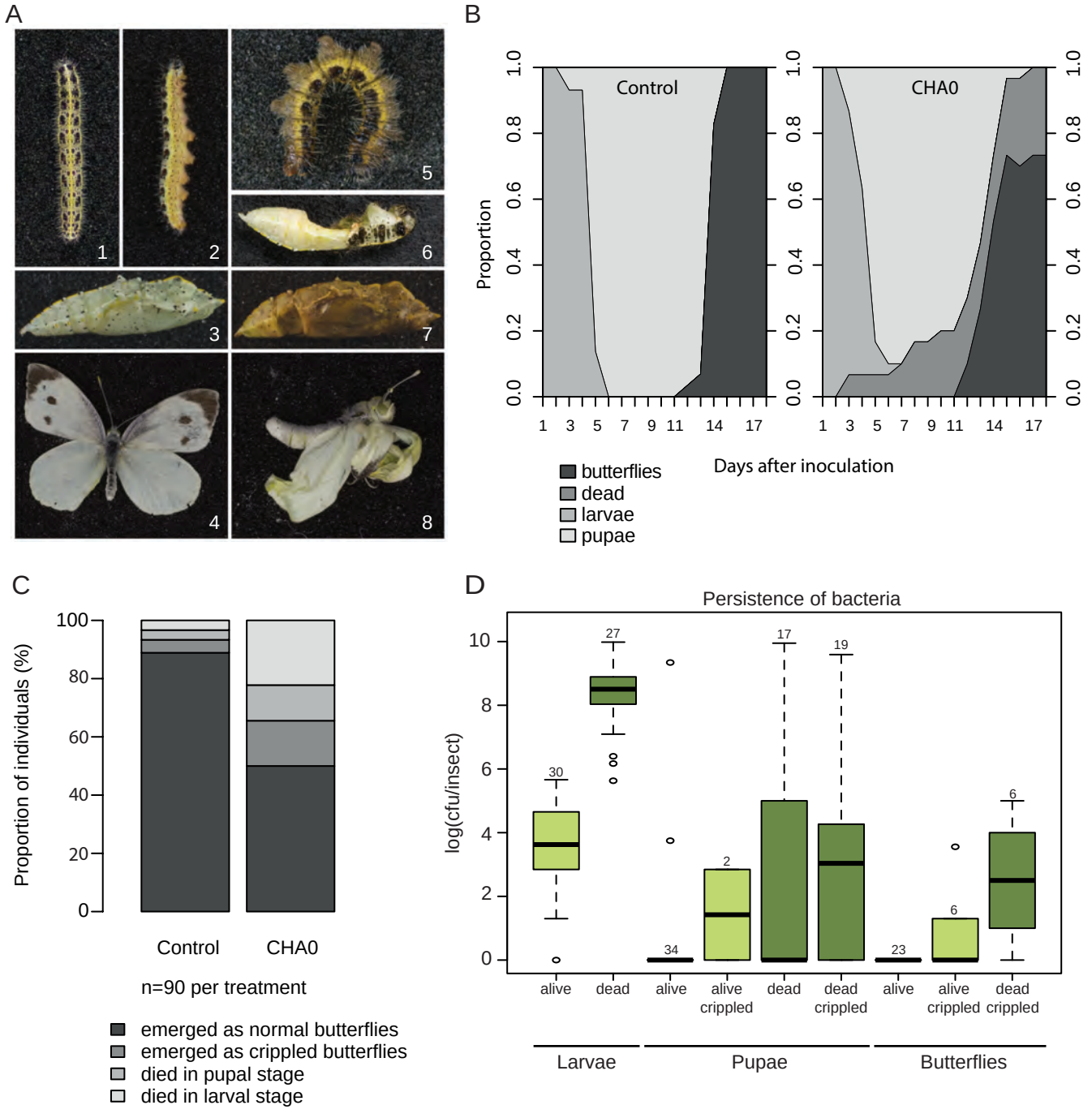
Gm^r, gentamicin resistance; Km^r, kanamycin resistance


A
B
C
D
pupation rate (%)
pupal size (mm²)
control
CHA0
PF
CMR12a
PCL1391
PITR2


A
100
80
60
40
20
0
transformation rate (%)
control
CHA0
larva to pupa
pupa to fly
B
6
5
4
3
2
1
0
log (cfu / insect)
17
22
22
3
larvae
pupae
flies
crippled flies
C
healthy
crippled
D
8
6
4
2
0
log (cfu / g roots)
3
13
control
CHA0


1
2
3
4
5
6
7
8
eggs


A
Proportion of individuals (%)
100
80
60
40
20
0
emerged as adults
died in pupal stage
died in larval stage
control
low
high
control
low
high
control
low
high
experiment 1
experiment 2
experiment 3
B
log (cfu / insect)
10
8
6
4
2
0
10
9
10
2
alive
dead
alive
dead
Larvae
Pupae
C
log (cfu / insect)
10
8
6
4
2
0
15
1
14
5
5
alive
dead
alive
dead
Larvae
Pupae
Adults
D
GL
ME
H
100 µm
F
H
B
GL
H
100 µm
H
H
GL
100 µm
J
H
GL
H
100 µm
E
B
GL
B
20 µm
G
ME
B
F
H
20 µm
I
H
GL
H
B
100 µm
K
H
H
GL
100 µm


A
1
2
3
4
5
6
7
8
B
Control
CHA0
Proportion
0.0
0.2
0.4
0.6
0.8
1.0
1 3 5 7 9 11 14 17
Days after inoculation
butterflies
dead
larvae
pupae
C
Proportion of individuals (%)
0
20
40
60
80
100
Control
CHA0
n=90 per treatment
emerged as normal butterflies
emerged as crippled butterflies
died in pupal stage
died in larval stage
D
Persistence of bacteria
log(cfu/insect)
0
2
4
6
8
10
30
27
34
2
17
19
23
6
6
alive
dead
alive
alive crippled
dead
dead crippled
alive
alive crippled
dead crippled
Larvae
Pupae
Butterflies

SUPPLEMENTARY INFORMATION

Supplementary Methods

Supplementary Figures

References

Supplementary Methods

Growing and inoculation of cauliflower plants

Cauliflower seeds (*Brassica oleracea botrytis* 'Walcheren Winter 5', Samen Mauser AG, Switzerland) were surface-sterilized as follows: seeds were placed for 2 min in 70% ethanol, thoroughly washed with sterile ddH_2O, subsequently placed for 30 min in 4% NaOCl, 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 (one seedling per pot) of which the lower two thirds were filled with autoclaved potting soil (TREF Go PP 7000 plant substrate, GVZ Rossat AG, Switzerland) and the upper third was filled with a mix of different fractions of quartz sand and vermiculite (Keel et al 1989). Each pot was amended with 10 ml of bacterial suspension (CHA0-*gfp2*, OD_{600} = 0.45; an OD_{600} of 0.125 contains about 10^8 cfu/ml) or water (control) and four trays, each containing twelve pots, were prepared for each treatment. Cauliflower plants were then grown for three weeks in a growth chamber with a 16-h day (20°C, 210 μmol m^{-2} s^{-1}), 8-h night cycle (18°C) and a relative humidity of 80%. For application of *Delia radicum* to cauliflower plants, a small piece of blue paper containing five freshly hatched larvae was placed next to the stem of the plants. Larvae that did not manage to enter the soil were replaced to ensure equal numbers of viable larvae on the roots.

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

Root colonization was assessed in four pots per tray of the *P. protegens* CHA0-*gfp2* treated plants and in all control pots to ensure that these were not contaminated with CHA0. Roots of cauliflower plants were washed on a sieve to remove adhering substrate. Then subsamples of the roots were placed in Eppendorf tubes containing 0.9% NaCl solution and incubated at 3°C over-night. Next, samples were shaken for 30 min at 1400 rpm on an Eppendorf thermomixer compact at 4°C. Serial dilutions were plated onto King's B (KB) agar plates (King et al 1954) supplemented with chloramphenicol (13 μg ml^{-1}), cycloheximide (100 μg ml^{-1}), ampicillin (40 μg ml^{-1}) and gentamicin (10 μg ml^{-1}) and plates were incubated at 27°C for two days. Colony forming units (cfu) were checked for expression of GFP with a Leica DM2500 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). In a few pots of the control treatment, a contamination by *P. protegens* CHA0-*gfp2* was observed. These samples were excluded from the analysis and emerging pupae not used for further experiments.

Transmission experiment with *Delia radicum*

Rapeseed seeds were sterilized and pre-germinated as described above for cauliflower seeds with the only difference that pre-germination lasted only one day instead of six days. Plastic beakers (500 cc) with a lid (Riwisa AG, Switzerland) were partly filled with 3 cm of autoclaved sand-vermiculite mix supplemented with 35 ml of Knop plant nutrient solution (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, 210 μmol m^{-2} s^{-1}), 8-h night cycle (18°C) and a relative humidity of 80%. Then, another 10 ml of Knop solution was added as well as sterile lids of Eppendorf tubes, one containing wet fly diet and one containing dry fly diet (Razinger et al 2014). Three to four pupae that had emerged from control or *P. protegens* CHA0-*gfp2* treatments in the cauliflower experiment (see above) were added per beaker in small sterile Erlenmeyer flasks (experiment 1) or sterile lids of Eppendorf tubes (experiment 2). In experiment 1, three of these transmission microcosms were established for the control treatment and fourteen for the CHA0-*gfp2* treatment. Of the latter one sample was excluded from the analysis, because no flies hatched. In experiment 2, nine transmission microcosms per treatment were established. Flies started to hatch after one day. Nine days later roots of rapeseed plants were checked for colonization by *P. protegens* CHA0-*gfp2* as described above for cauliflower plants. Roots of plants grown in the same beaker were pooled for analysis.

Experiments with *Otiorhynchus sulcatus*

Larvae of *O. sulcatus* and strawberry plants were kindly provided by Matthias Lutz (ZHAW Wädenswil, Switzerland). Root balls of two months old strawberry plants ('Elsanta', Näppbrunnenhof, Switzerland) were incubated for 10 min in a cell suspension of *P. protegens* CHA0 (OD_{600} = 0.0125) or in sterile water for control treatments and in the following planted in pots containing potting soil (TREF Go PP 7000 plant substrate, GVZ Rossat AG, Switzerland). Fifteen (experiment A) or ten (experiment B) last-instar larvae of *O. sulcatus* were added per pot. Plants were kept at 18°C day temperature, 15°C night temperature, 60% humidity and a 16-h day, 8-h night cycle in a growth chamber. After one month, pupae were harvested and either directly extracted or maintained until adults emerged. Three pupae and three adults of both treatments were surface sterilized (30 s 70% ethanol, rinsed in 0.9% NaCl solution) and homogenized in sterile 0.9% NaCl solution with a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland). The resulting suspensions were serially diluted and plated onto KB agar plates supplemented with chloramphenicol (13 μg ml^{-1}), cycloheximide (100 μg ml^{-1}) and ampicillin (40 μg ml^{-1}). 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. The experiment was conducted twice.

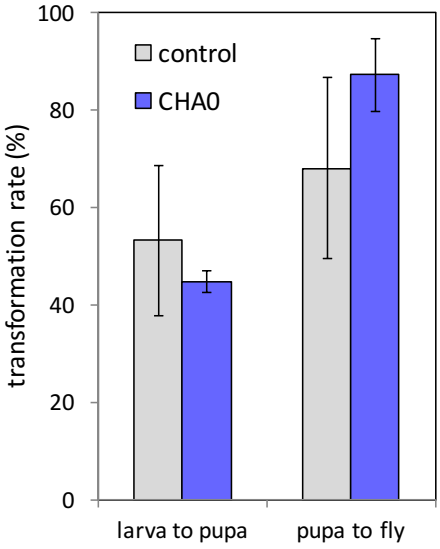
Survival and colonization experiments with *Plutella xylostella*

The experiments with *Plutella xylostella* were conducted as detailed in Flury et al (2017). 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, experiment 3 with 64 larvae per treatment. Larvae and pupae were considered dead when they did not react to poking. From each treatment, five individuals per

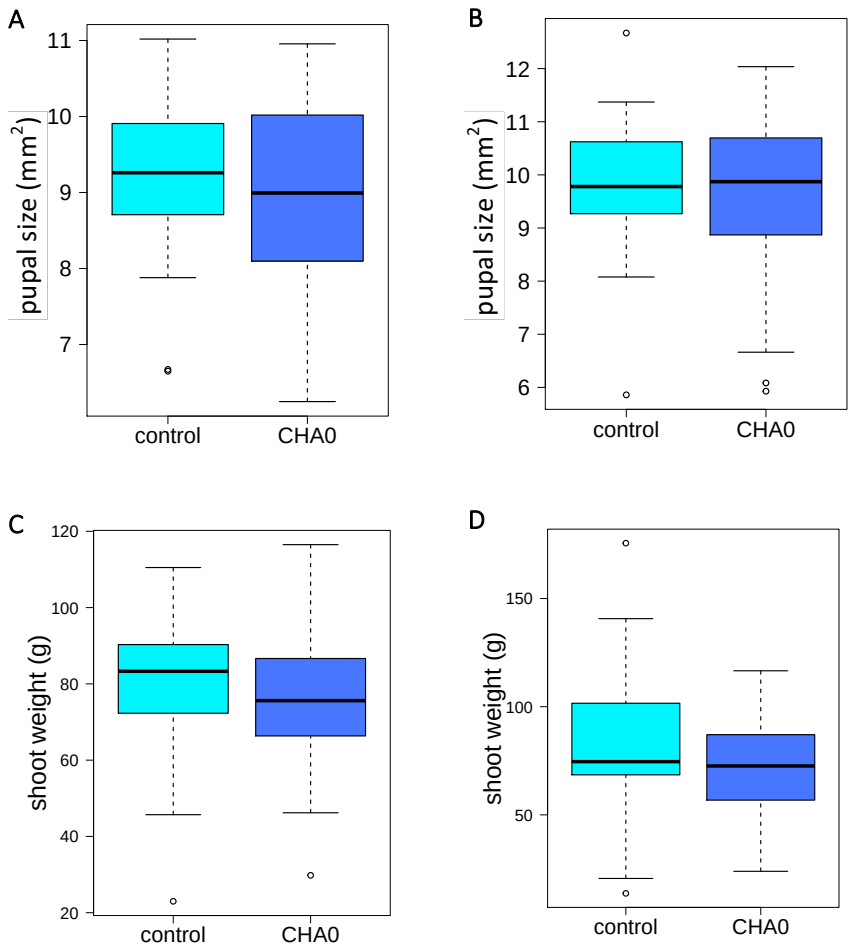
developmental stage (alive and dead) or as many as available were extracted as described under 'Assessment of bacterial colonization rates' in the main paper.

Microscopy

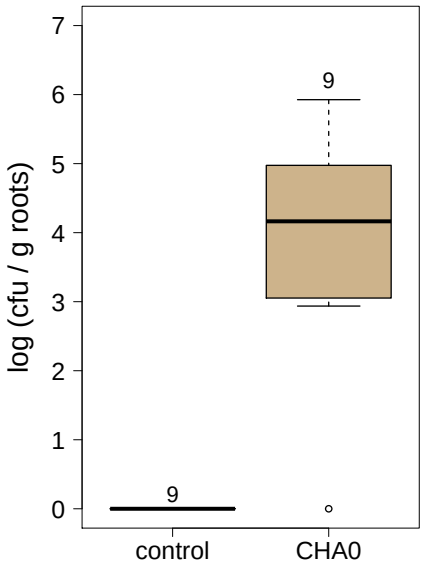
For microscopy, twelve one-week-old *P. xylostella* larvae were kept together in a Petri dish which was lined with a wetted filter paper and contained four pellets of artificial diet. For the bacterial treatments diet was inoculated with 10 µl of bacterial suspension of OD_{600} = 10, which corresponds to about 8 x 10^7 cells. Larvae were collected at different time points after infection (a total of 27 infected larvae were investigated by microscopy), were killed by exposure to ethyl acetate, and subsequently fixed for 24 h in Duboscq-Brazil's alcoholic Bouin's (saturated alcoholic solution of picric acid, formaldehyde, glacial acetic acid, 10:4:1 [vol/vol]). After dehydration in ascending concentrations of ethanol, larvae were embedded in Histosec (Merck, Darmstadt, Germany). Embedded larvae were cut into serial sections of 6 µm, mounted onto microscope slides and cleared from Histosec with xylene. For histopathology analysis, sections were stained with Heidenhain's iron hematoxylin, counterstained in erythrosine and examined in a Leica photomicroscope, model DMRB (Leica, Wetzlar, Germany). To be able to identify the applied bacteria, the GFP tagged variant *P. protegens* CHA1176 (Table 1) was used instead of wild type CHA0. However, fixation in Duboscq-Brazil's alcoholic Bouin's destroys intrinsic GFP fluorescence and immunofluorescence microscopy was needed to specifically detect the bacteria. To allow access of the antibodies to the intracellular GFP, tissue sections were boiled for 30 min at 90°C in 10 mM sodium citrate, washed in PBS and blocked in 1% BSA, 0.3%Triton X-100 in PBS as described by Benjamin et al (2013). Sections were then incubated in monoclonal mouse anti-GFP IgG (1:500, Roche, Switzerland) for 1 h at room temperature and subsequently over-night at 4°C. After washing three times in PBS, sections were incubated with donkey anti-mouse IgG-FITC (1:200, Dianova, Germany) for 4 h at room temperature and were examined with a Leitz Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany). All images were captured with an SIS ColorView II camera (Soft Imaging System GmbH, Münster, Germany).



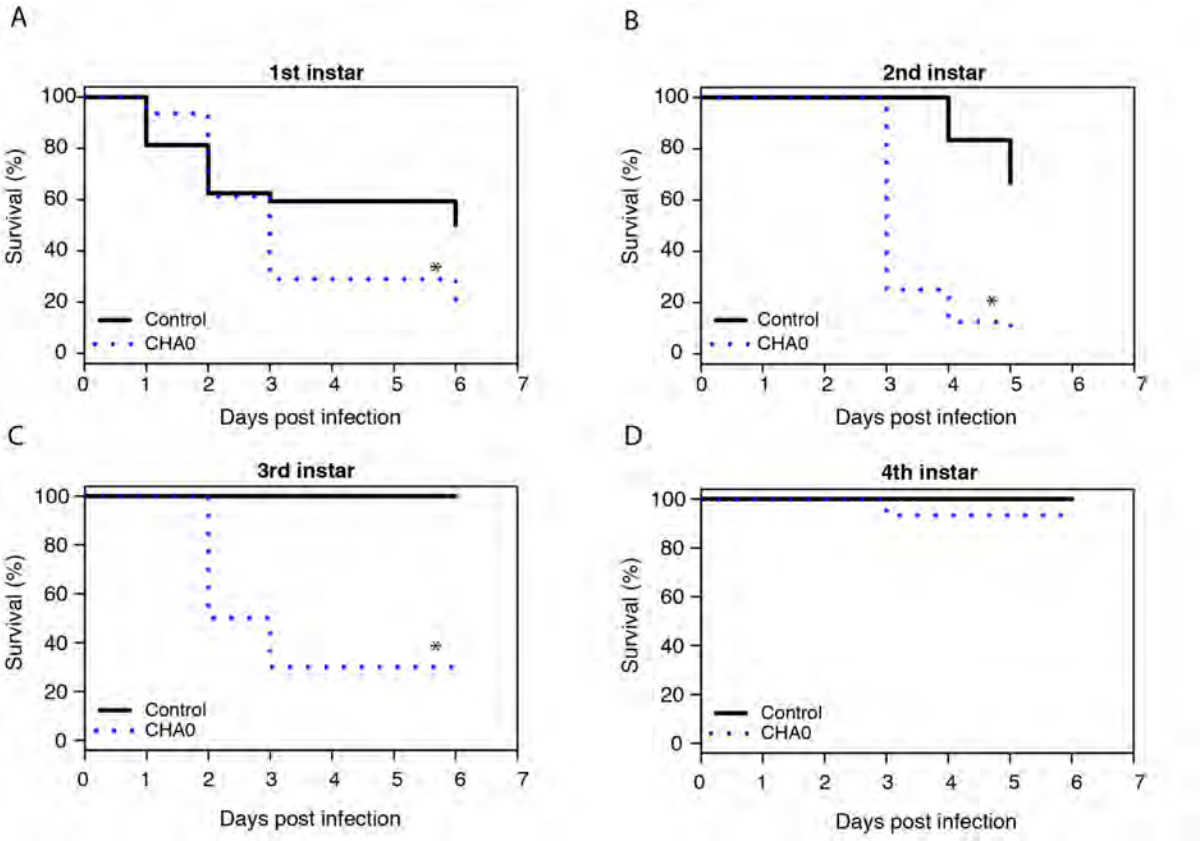
Supplementary Figure S1. *Pseudomonas protegens* CHA0-*gfp2* does not affect survival of *Delia radicum*. This experiment is a repetition of the one depicted in Figure 2A. Five freshly hatched *D. radicum* larvae were added to cauliflower plants (four trays per treatment, each containing twelve pots)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



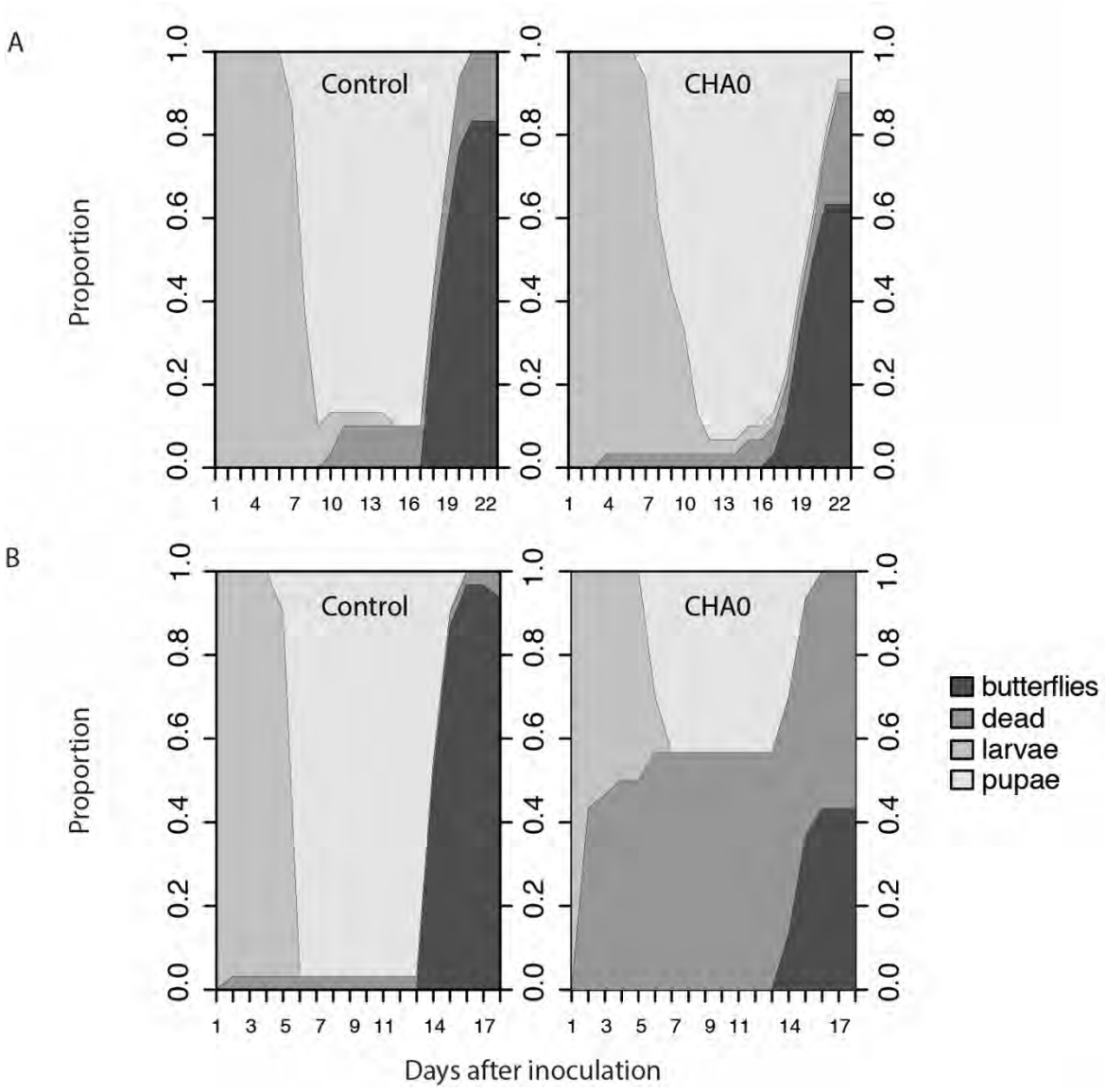
Supplementary Figure S2. *Pseudomonas protegens* CHA0-*gfp2* does neither reduce pupal size of *Delia radicum* nor increase the shoot weight of cauliflower plants infested with the insect. Roots of cauliflower plants were inoculated with a cell suspension of *P. protegens* CHA0-*gfp2* (CHA0) or amended with water (control) at planting (n = 48). Three weeks later, five freshly hatched *D. radicum* larvae were added and let feed on the roots until pupation. Then size of emerged pupae **(A, B)** as well as shoot weight of cauliflower plants **(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 1 and 2 are depicted in **(A, C)** and (**B, D)**, respectively.



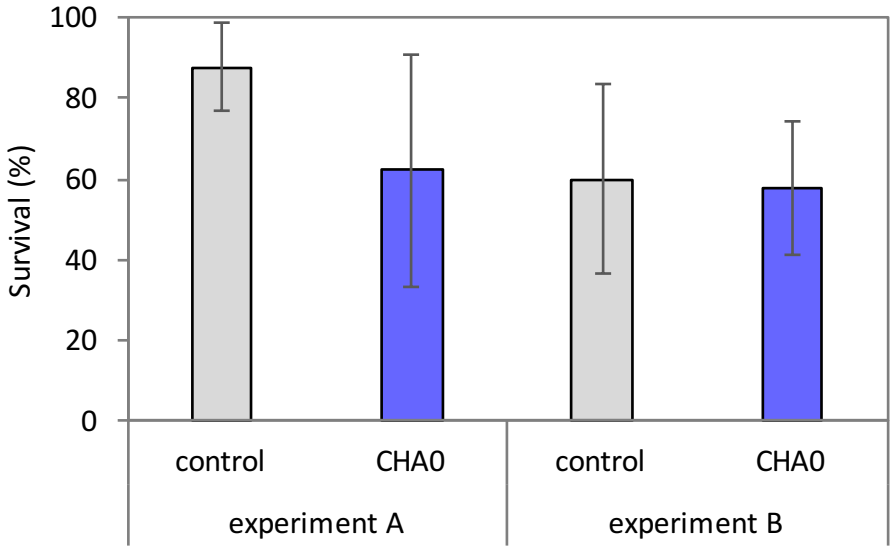
Supplementary Figure S3. *Pseudomonas protegens* CHA0 taken up by root-feeding *Delia radicum* larvae can be dispersed to a new host plant. This experiment is a repetition of that depicted in Figure 2D. Colonization levels of *P. protegens* CHA0-*gfp2* on roots of rapeseed plants. Plants were exposed for nine days to flies, which as larvae had fed on roots of control or CHA0-*gfp2* treated cauliflower plants, i.e. the previous plant host. In the CHA0 treatment roots in eight out of nine systems became colonized with *P. protegens* CHA0-*gfp2*. Numbers above boxes indicate sample size.



Supplementary Figure S4. First, second and third instar *Pieris brassicae* larvae, but not fourth instar larvae are highly susceptible to an infection with *Pseudomonas protegens* CHA0. Kaplan-Meier survival graphs of different larval stages of *P. brassicae* treated with *P. protegens* CHA0. First **(A)**, second **(B)**, third **(C)** and fourth **(D)** instar larvae (n=24 to 32) of *P. brassicae* were fed with a pellet of artificial diet inoculated with ~10^8 bacteria or amended with 0.9% NaCl solution (Control). Only larvae that consumed the entire piece of diet were included in the analysis. Asterisks indicate significant differences according to a Log-Rank test ($p \leq 0.05$, Survival Package in R).



Supplementary Figure S5: Impact of *Pseudomonas protegens* CHA0-*gfp2* on development and mortality of *Pieris brassicae* over time. These experiments are two repetitions **(A and B)** of the one depicted in Figure 5B. Thirty fourth-instar *P. brassicae* larvae were fed with artificial diet inoculated with 10 µl of a bacterial suspension of OD_{600}=20 or amended with 0.9% NaCl solution (control). The different development stages of the insects were monitored during 17 or 23 days until the butterflies emerged from the pupae.



Supplementary Figure S6. *Pseudomonas protegens* CHA0 does not affect survival of *Otiorhynchus sulcatus* larvae. The roots of strawberry plants (one plant per pot, six and five pots per treatment in experiment A and B, respectively) were either inoculated with *P. protegens* CHA0 (CHA0) or mock (control). Fifteen (experiment A) or ten (experiment B) *O. sulcatus* larvae were added per pot. After one month insect survival per pot did not significantly differ between control and CHA0 treatment in both experiments ($p < 0.05$; Student's t test). Error bars depict standard deviations of the mean.

References

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