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

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Abstract

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

Introduction

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

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

The first insecticidal trait discovered was the Fit toxin [17] typically produced by strains belonging to the *P. protegens* and *P. chlororaphis* species [16]. The contribution of this protein toxin to oral and systemic insecticidal activity and its tight insect host-dependent regulation were studied in some detail in *P. protegens* type strain CHA0 [15, 20, 26, 27]. Fit toxin production only partially explains toxicity of these bacteria as *fit* deletion mutants retain some toxicity [15, 20]. Studies on *P. protegens* CHA0 and other *P. protegens/P. chlororaphis* strains related insecticidal activity and host persistence to additional factors, including type VI secretion components [28], chitinase and phospholipase C [16], hydrogen cyanide [29], the cyclic lipopeptide orfamide [29, 30], the toxins rhizoxin [31] and IPD072Aa [32], and specific lipopolysaccharide O-antigens [33]. *P. protegens/P. chlororaphis* strains can also cause nonlethal infections [18, 22, 23, 31]. Even if the infection does not kill the insect after oral uptake, strains such as CHA0 can persist until pupal and imago stages, thus affecting the insect development as shown for *Delia radicum*, *Plutella xylostella* and *Pieris brassicae*, and be transmitted to new host plants by *D. radicum* [23]. Therefore, the ability of *P. protegens* to colonize cross-kingdom insect and plant hosts is impressively demonstrated by work on the model strain CHA0. However, it remains largely unknown what specific traits underlie cross-kingdom host colonization and how plastic responses including transcriptional remodeling contribute to switching between hosts.

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

**Material and methods**

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

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

**Grace’s insect medium (GIM) and lysogeny broth (LB)**

*P. protegens* CHA0 was grown on KB+++ agar for two days. Single colonies were transferred to LB [36] or GIM (Sigma Aldrich, MO, USA) and grown to OD₆₀₀ = 1.74–1.86 (−1.5 × 10⁹ cells/ml) at 24 °C while shaking at 180 rpm. Four milliliters of cultures were centrifuged (7500 rpm) and pellets used for RNA extractions.

**Wheat roots**

Root colonization assays were performed as described in de Werra et al. [37] and further explained in Supplementary Methods. Briefly, pre-germinated seeds of spring wheat, variety Rubli, were inoculated with 1 ml of a suspension containing 10⁸ CHA0 cells/ml and placed into seed germination pouches. Plants were grown at 22 °C and 60% humidity with a 16/8 h day/night cycle. After 1 week, roots of 99 wheat plants per replicate were harvested, shaken in 0.9% NaCl, the resulting suspensions were centrifuged and pellets containing bacteria were pooled for RNA extraction.

**P. xylostella gut (oral infection)**

*P. xylostella* eggs were kept before and during the experiment at 25 °C, 60% relative humidity and a 16/8 h day/night cycle with 162 μmol m⁻² s⁻¹. Second instar *P. xylostella* were fed with pellets of artificial diet spiked with 4 × 10⁶ CHA0 cells or NaCl 0.9% (control) as previously described by Flury et al. [29] and further explained in Supplementary Methods. For each replicate 120 treated alive larvae were collected 24 or 36 h post-feeding, surface disinfected and homogenized and homogenates were pooled. Thirty to sixty-five larvae per treatment were used for assessing survival over time. In addition, the resident cultivable microflora of untreated insects was assessed by growing the extracts on LB media plates at 18, 24, and 37 °C for 1 week.

**G. mellonella hemolymph (hemocoel injection)**

Seventh instar *G. mellonella* larvae were injected with 2 × 10³ CHA0 cells or 0.9% NaCl solution as previously described by Flury et al. [29] and further explained in Supplementary Methods. After 24 h, 55 alive non-melanized larvae per replicate were surface disinfected, one leg was cut and the hemolymph collected. Thirty to fifty larvae per treatment were used to assess survival.
**RNA extraction and sequencing**

The range of total numbers of CHA0 cells used for sequencing were: LB, 5.6–5.9 \times 10^7; GIM, 5.5–5.9 \times 10^7; wheat, 5 \times 10^6–2 \times 10^{10}; P. xylostella 24 h, 2.8 \times 10^6–4.1 \times 10^7; P. x. 36 h, 2.4 \times 10^6–1.6 \times 10^8; and G. mellonella, 8.2 \times 10^7–2.2 \times 10^9 cells. RNA from insect and media samples was extracted using the GENEzol Reagent (Gen-ea International, Taiwan) and from wheat root samples using the RNeasy Plant Mini Kit™ (Qiagen, Germany) without bead-rupture. All extracts were treated with DNase from the RNeasy Mini Kit™ (Qiagen, Germany). RNA quality was assessed using the 2200 Tapestation (Agilent, CA, USA) and Nanodrop 2000 (Thermo Scientific, MA, USA). For details, see Supplementary Table S1. Libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit. The RiboZero Bacteria kit was used for medium samples, RiboZero Epidemicology for insect samples and RiboZero Plant Root for wheat samples to remove bacterial, insect and plant RNA, respectively (illumina, CA, USA). Samples were sequenced using Illumina Next-Seq500 single-end 81 bp sequencing (illumina, CA, USA) at the Genomics Facility BSSE in Basel, Switzerland.

**Bioinformatics analysis**

**Read trimming, alignment, and normalization**

Raw reads were quality trimmed and filtered for adapter contamination with Trimmomatic 0.36 [38]. Processed reads were aligned against the P. protegens CHA0 genome (NCBI entry number LS999205.1, [39]) using STAR 2.3.5a [40]. Mapped reads were counted with featurecounts 1.5.3 [41] using the stranded option and normalized with the TMM (trimmed mean of M values) method [42] of the edgeR 3.26.6 package [43] in R 3.6.0 (www.r-project.org). Genes with <1 count per million (CPM) in the four biological replicates were discarded. Normalized CPM were used for: (1) a multidimensional scaling analysis; (2) a transcriptome profile analysis with the K-means algorithm. The optimal number of clusters was assessed with the sum of square error method, which showed that six clusters can optimally predict the different transcription patterns; (3) a differential gene expression (DGE) analysis following the general linear model from edgeR package [43, 44]. The differentially expressed genes were determined using “glmFit” function with a Benjamin–Hochberg FDR correction for false positives; (4) Log10 transformation and heatmap generation with heatmap.2 package (https://CRAN.R-project.org/package=gplots).

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

**Ortholog analysis**

Whole protein sequences of 97 Pseudomonas species (Supplementary Table S2) were compared using Ortho-Finder 2.3.3 [48] in an orthologue protein analysis with the default settings. Results were then filtered for chosen categories of proteins. The tree output was represented using FigTree 1.4.4 (http://tree.bio.ed.ac.uk/).

The detailed RNA-seq Script is placed in Supplementary Material 2.

**RT-qPCR**

In order to verify the RNA-seq results, the expression of selected genes *pap*, *chiD*, *pltA*, *tpsA2*, *tpsA4*, and the PPRCHA0_1961 IS3 transposase gene in the investigated environments/hosts were quantified using RT-qPCR as described in Supplementary Methods, and Supplementary Tables S3 and S4.

**Role of two-partner secretion (TPS) proteins**

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

**Statistics of insect assays**

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

**Results and discussion**

We analyzed the transcriptomic plasticity enabling *P. protegens* CHA0 to colonize lepidopteran larvae and wheat roots. These constitute two very different ecological niches in which CHA0 is known to establish pathogenic and beneficial interactions, respectively. We used the CHA0 strain to inoculate wheat roots, feed *P. xylostella* larvae or inject the hemocoel of *G. mellonella* larvae.

We analyzed the transcriptome of CHA0 after 1 week on wheat roots, when bacteria had established population sizes
of $10^5$–$10^6$ CFU/mg dry root (Fig. 1b). At this time point, where roots elongate fast, we expect the transcriptome to represent the whole spectrum of bacterial root colonization with already established microcolonies on older roots and continuing growth towards the root tips. In addition, the absence of resident microflora from the soil or the plant, allowed us to observe transcriptomic variation exclusively in response to the plant.

*P. xylostella* was selected as model to study the progress of insect gut infection 24 and 36 h after feeding. Previous microscopy studies showed that, 1–2 days after feeding *P. xylostella* with treated pellets, CHA0 could only be found in the microvilli of the gut cells and, 3 days after feeding, the insect hemocoel was already heavily colonized by CHA0 [23]. In addition, *P. xylostella* larvae were shown to only harbor up to 100 cultivable CFU (including bacteria and fungi) of resident microorganisms per larvae at the moment of feeding with CHA0 (Supplementary Fig. 1c). In our study, at 24 h, the larvae showed no disease symptoms yet and were colonized by $10^4$–$10^5$ CFU/mg larvae (Fig. 1a, b). At 36 h bacterial loads were ten times higher, some treated larvae started to die and the remaining were smaller and darker than non-treated larvae, indicating the start of bacterial transmigration from gut to hemocoel (Fig. 1a, b). Final mortality assessed in non-extracted *P. xylostella* was 90.6–98.4% (Fig. 1a and Supplementary Fig. S2). When CHA0 transmigrates into the hemocoel, it causes a systemic infection and eventually kills the insects.

Next, we wanted to understand the transcriptomic response in a pure hemolymph environment excluding transmigration factors. Therefore, we injected CHA0 directly into the hemocoel of the bigger insect model

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**Fig. 1** Toxicity (a), cell density (b), and MDS transcriptome analysis (c) for *Pseudomonas protegens* CHA0 colonizing different hosts and media. The “Wheat” samples correspond to wheat-roots 1 week after inoculation, “*P. xylostella* 24/36 h” to *Plutella xylostella* 24 and 36 h after oral infection; “*G. mellonella*” to *Galleria mellonella* hemolymph 24 h after hemocoel injection; “LB” to lysogeny broth, and “GIM” to Grace’s insect medium. a) Survival of *P. xylostella* larvae after exposure to artificial diet pellets spiked with $4 \times 10^6$ CHA0 cells (top) and of *G. mellonella* larvae upon injection of $2 \times 10^5$ CHA0 cells (bottom). One representative experiment with 64 (*Plutella*) and 30 (*Galleria*) larvae is shown. Time points where insects were sampled for RNA extraction are indicated with arrows. b) Bacterial densities at collection time points. Boxplots are created from four replicates per environment and show CFU per mg of root dry weight (wheat), CFU per mg of larvae (24/36 h), CFU per µl hemolymph (*G. mellonella*) and CFU per ml medium (GIM, LB). c) Multidimensional Scaling (MDS) analysis was performed with four replicate CHA0 transcriptomes per environment. The different replicates of the different biological samples are numbered from 1 to 4.
G. mellonella and extracted RNA 24 h later when the larvae were still alive, non-melanized and inoculant cell numbers had reached \(10^5\) CFU/µl hemolymph (Fig. 1b). CHA0 killed 93.3–100% of G. mellonella larvae within 48 h (Fig. 1a and Supplementary Fig. S2). We used the G. mellonella injection model because to inject P. xylostella larvae without causing harm and to extract enough hemolymph for RNA sequencing without disrupting the gut is very difficult due to their small size.

In addition to CHA0 transcriptome remodeling on insect hosts, we analyzed the bacterial response to an insect-simulating culture medium without the influence of host immune responses i.e., GIM. Finally, the transcriptome of CHA0 was analyzed when growing in a rich culture medium (Lysogeny broth).

We obtained \(10^8\)–\(10^9\) reads from RNA Illumina NextSeq sequencing for each sample and quantified expression levels of CHA0 gene models (Supplementary Table S1). We performed a multidimensional scaling analysis to distinguish CHA0 transcriptomes according to the colonized host or culture condition (Fig. 1c). All transcriptomes were differentially separated except for the P. xylostella at 24 and 36 h due to the differences in infection progression across the biological samples. The first principal component axis of Fig. 1c separates the transcriptomes from culture media from those obtained from living hosts. This is probably due to the fact that bacteria need to actively colonize both wheat and insect hosts even though bacteria do not establish a pathogenic interaction on roots. This may explain the position of the wheat transcriptome between the culture media and the insect backgrounds. The second principal component separates P. xylostella gut and G. mellonella hemolymph samples (Fig. 1c). Some differences in gene expression between gut and hemolymph environments may be due to the different lepidopteran species used. However, CHA0 was shown to multiply in and kill larvae of different lepidopteran species following a similar pattern [15, 16, 20, 23, 26, 28]. Figure 1c also shows the transcriptome plasticity within the same insect species probably due to the implication of different bacterial factors at different phases in the infection.

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

The clustering analysis revealed distinct expression profiles for each of the hosts and culture condition (Fig. 2a). This implies that the CHA0 transcriptome changes drastically according to the colonized plant or insect host. We also monitored pronounced differences between the P. xylostella gut and the G. mellonella hemolymph, which indicates that a different set of genes is active in different insect compartments. But we cannot exclude that some of the observed differences might be due to the use of two different insect species.

We found that general metabolic processes and genes related to organic compound biosynthesis were upregulated on wheat roots (Figs. 2b and 3a). Genes related to nucleotide and protein synthesis were downregulated during insect colonization compared to wheat roots (Fig. 3a). This might indicate that at the chosen time points the bacterium was more metabolically active on the roots e.g., colonizing growing root zones, whereas bacterial growth may have been restricted in the gut. Though, our study reflects only specific moments in time and we cannot generally conclude that proliferation on the roots was higher than proliferation in the gut. Observed differences in gene expression could be caused by host-derived factors or by differences in growth rate or population sizes. This implies that the CHA0 transcriptome changes significantly depending on the colonized plant or insect. We also found pronounced differences between P. xylostella gut and G. mellonella hemolymph suggesting that a different set of genes is used for different insect compartments. However, the use of two insect species may influence on the observed differences.

To successfully reach root zones where exudates are released and to outcompete other organisms, the bacteria need to actively move [49–51]. Motor activity-related genes were expressed under all conditions but especially upregulated on wheat roots as shown by clustering, DGE and heatmap analyses (Figs. 2b, 3a, b and Supplementary Fig. S3). This confirms the relevance of flagella for wheat root colonization. Interestingly, the reb genes required for R-body synthesis were upregulated on wheat roots and repressed in both insect compartments (Fig. 4), which supports the lack of differences in insecticidal activity between a ΔrebB1 mutant and wildtype CHA0 in oral and injection assays [16]. R-bodies disrupt membranes and deliver toxins in several bacterial genera including Pseudomonas. In Azorhizobium caulinodans, the R-bodies have also been related to Paramecium killing and cell-disruption in the legume Sesbania rostrata [52–54].

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

The gut is a challenging environment for exogenous bacteria as they have to compete with the resident microflora and to overcome the insect immune response e.g., antimicrobial peptides (AMPs) and reactive oxygen and nitrogen species (ROS and RNS) [55]. The P. xylostella expression profiles reveal upregulation of several genes, which might help CHA0 to cope with these menaces in the gut. The P. xylostella 24 h transcription profile main cluster
harbors most of the genes related to amidase activity (Fig. 2). Amidases cleave proteoglycans that trigger host immune response thereby helping bacteria to avoid recognition [55–57]. We suggest a similar mechanism in our model. At 36 h after feeding, we found that genes related to the transport of nitrogen and organic substances
In order to persist, attach and breach the gut epithelium, CHA0 must successfully compete against the resident gut bacteria. CHA0 competes in the rhizosphere by producing a variety of antimicrobial compounds, which contribute to rhizosphere competence and the suppression of soilborne pathogens [5]. Among the tested hosts, all of the biosynthetic genes involved in the production of the broad-spectrum antimicrobials hydrogen cyanide, 2,4-diacetylphloroglucinol, and pyrrolnitrine were expressed in *P. xylostella* and expressed at low levels on wheat roots (with the exception of 2,4-diacetylphloroglucinol which was highest expressed on roots), but were not expressed in the *G. mellonella* hemolymph. Although the expression of hydrogen cyanide is low in our study, it has been previously shown to influence insect survival when CHA0 is injected into the *G. mellonella* hemolymph [29]. Although the expression of pyoluteorin biosynthetic genes was not detected on the roots of any tested plants [37], they were expressed in *P. xylostella* (Supplementary Fig. S3). Hence, pyoluteorin might play a more important role in persistence in insect hosts than on plant roots. Combined, our results support that antimicrobials are not only used for competition against microorganisms in the rhizosphere but also during colonization of an insect. We think that the expression of antimicrobials was mainly modulated by the insect background and less by the presence of resident microorganisms in the *Plutella* gut because the analysis of control larvae revealed a very low microbial background cultivable on LB (Supplementary Fig. S1). Likewise, CHA0 expresses antimicrobials on roots, even in the absence of other microorganisms [37].

The type VI secretion system (T6SS) has been related to pathogenicity and bacterial competition in the insect gut. In CHA0, the *vgrG1a* and *vgrG1b* genes encoding distinct T6SS spikes and *rhsA* and *ggh1* encoding respective associated effectors with predicted nuclease activity were demonstrated to contribute to invading *P. brassicae*. These genes play a role in the ability of CHA0 to compete with the gut microflora and impact on its composition [28]. In our study, these genes were expressed in both insect models (Fig. 4). This underlies the importance of these T6SS components during competitive host invasion [66–68]. Interestingly, the expression of *vgrG1b* and its associated effector gene *ggh1* was higher in the *P. xylostella* gut than in the *G. mellonella* hemolymph. But we found the opposite for *vgrG1a* and *rhsA* (Fig. 4) indicating that T6SS-mediated competition is important not only in the gut but also at a later infection stage in the hemolymph.

Expression patterns of *pap*, *chiD*, *pltA*, and the PPRCHA0_1961 IS3 transposase analyzed by qPCR showed the same tendencies as in the RNA-seq with significant differences between environments (*p* < 0.05) (Supplementary Fig. S4).

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**Fig. 2** Transcription profiles of *Pseudomonas protegens* CHA0 during the colonization of different hosts or environments. a Normalized counts per million (CPM) of *P. protegens* CHA0 transcriptomes obtained using the K-means clustering method. Genes clustered in six different groups according to different hosts/media. Standard errors for four biological replicates show small variations among the genes included in each cluster. The number of genes that belong to the main cluster of each transcription profile are indicated. From each cluster, those genes with a gene ontology (GO) annotation were used in the enrichment analyses presented in b. Genes related to oxidoreductase activity proteins (Fig. 3a). This supports invasion competences through oral and systemic infection; infection; after hemocoel injection, insect medium.

were specifically upregulated (Fig. 2b, *P. xylostella* 36 h). This might be related to the use of nitrogenous compounds emerging from the interaction of ROS and RNS during the insect immune response [58]. The combined evidence from the *P. xylostella* 24/36 h transcriptomic responses compared to wheat root transcriptomes showed upregulation of coding genes related to oxidoreductase activity proteins (Fig. 3a). This could be related to the bacterial defense against ROS produced by the insect host. Among the most upregulated genes in the *P. xylostella*-wheat comparison, were *pap* and PPRCHA0_1961 encoding a polyphosphate kinase (PPK) and a predicted “copy-paste” transposase, respectively (Fig. 4 and Supplementary Table 6). PPKs have been related to motility, quorum sensing, biofilm formation, and virulence of *P. aeruginosa* and regulation of stress response in *Campylobacter jejuni* [59–63]. We hypothesize that *pap* might play a similar role in CHA0 insect pathogenesis. The PPRCHA0_1961-encoded transposase might perform genomic rearrangements which are important for bacterial adaptation and pathogenesis as shown for *P. aeruginosa* with another transposable element family [64, 65].

The insect immune response also triggers the production of AMPs that kill invasive bacteria [55]. The dominant short O-antigen polysaccharide (O-PS) encoded by the OSA cluster confers resistance to insect AMPs in CHA0 [33]. This supports invasion competences through oral and systemic insecticidal activity of the bacterium. Of the four CHA0 O-PS clusters [33], OSA and OBC3 (which encodes the major long O-antigen of CHA0) were expressed in all our backgrounds. Interestingly, OBC1 and OBC2 were only expressed in the *P. xylostella* gut (Supplementary Fig. S3). It remains unknown whether OBC1 and OBC2 might also play a role in avoiding resistance to or recognition by the insect immune system.
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Functions underlying transmigration from the gut lumen into the hemocoel

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

Orfamide A, the chininase and the phospholipase C encoded by ofaABC, chiD, and plcN, respectively, showed the highest expression in P. xylostella when compared to the other environments (Fig. 4 and Supplementary Fig. S3). In previous studies, CHA0 mutants lacking any of these genes had reduced activity in oral P. xylostella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assays, but not upon injection into G. mellonella feeding assay. The four tpsA genes showed very different expression profiles across the examined environments (Fig. 5b). While tpsA2 was equally expressed in all examined hosts, the other three variants were upregulated in insects when compared to wheat roots. tpsA3 was similarly expressed in both insect models, in comparison to tpsA1 which was upregulated during P. xylostella infection and tpsA4 which was highly upregulated in G. mellonella hemolymph. Interestingly, tpsA4 was among the 20 most upregulated genes in G. mellonella when compared to P. xylostella (Supplementary Table S6).

In order to assess the importance of TpsA toxins for insect infection, we tested tpsA knockout mutants in feeding assays with P. xylostella and in injection assays with
G. mellonella. The deletion of tpsA4, which was highly expressed in an insect background but not on wheat roots, resulted in significantly reduced insecticidal activity in two out of three feeding experiments. A deletion mutant of tpsA2, which was expressed in all tested hosts, led to significantly reduced insecticidal activity in one out of three feeding experiments (p < 0.05) (Fig. 5c and Supplementary Fig. S5a, b). In addition, a tpsA4 deletion mutant showed significantly slower mortality when injected into the G. mellonella hemocoel in two experiments (p < 0.05) (Fig. 5d and Supplementary Fig. S5c).

Furthermore, we confirmed the expression patterns of tpsA2 and tpsA4 by qPCR showing the same tendencies as found in the transcriptomic analyses with significant differences between environments (p < 0.05) (Supplementary Fig. S4).

The functions of the CHA0 TPS-secreted toxins are still unknown, but these toxins may play similar roles as the P. aeruginosa toxin ExLA. TpsBA1-3 may be involved in gut colonization, adhesion to tissues, bacterial competition, and the disruption of the gut epithelium. The high expression of TpsBA4 in the hemolymph suggests that the protein could be involved in the defense against insect immune reactions e.g., by triggering hemocyte cell death. Our model of TPS interactions are supported by the findings that CHA0 mutants lacking tpsA4 are largely impaired in macrophage killing [81].

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

During the first phase of gut infection, CHA0 shows limited growth and metabolism. In contrast, we found an upregulation of structural ribosome constituents and nucleic acid synthesis in the G. mellonella hemolymph (Figs. 2b and 3c). The pairwise comparison revealed a general upregulation of genes involved in proliferation and metabolic activities in the G. mellonella hemolymph compared to the P. xylostella gut (Fig. 3c). In contrast, oxidoreductase and catalase activity were downregulated compared to P. xylostella and wheat (Fig. 3c, b). These differences indicate that the hemolymph is a less stressful environment and that CHA0 has enough nutrients allowing rapid proliferation leading to the systemic infection and ultimately death of the insect. However, oxidoreductase functions were upregulated in the

**Color Key**

![Color Key](image)
**Fig. 5** Two-partner secretion (TPS) systems in *Pseudomonas protegens* CHA0: domain analysis (a), expression profiles in relation to different hosts (b), and contribution to insecticidal activity (c).

**a** Domain analysis of the secreted protein with HMMER database comparing *Bordetella pertussis* protein FhaB, *Pseudomonas aeruginosa* PA7 protein ExLA and *P. protegens* CHA0 proteins TpsA1, TpsA2, TpsA3, and TpsA4 (PPRCHA0_0168-169, PPRCHA0_0625-0626, PPRCHA0_1574-1575, and PPRCHA0_4277-4278, respectively). The signal peptide and TPS domains are used to interact with the transporter protein for membrane translocation; the filamentous hemagglutinin 1 attaches to the host-cell and the filamentous hemagglutinin 2 translocates the PT-VENN domain into the host; DUF637 is common to hemagglutinins but its function is still unclear [83].

**b** Heatmap showing the normalized expression values for genes related to the four complete two-partner secretion systems in *P. protegens* CHA0 colonizing different hosts. The “Wheat” sample corresponds to wheat-roots 1 week after inoculation, “*P. xylostella* 24/36 h” to *Plutella xylostella* 24 and 36 h after oral infection, “*G. mellonella*” to *Galleria mellonella* hemolymph 24 h after hemocoel injection.

**c** Survival of *P. xylostella* larvae after exposure to artificial diet pellets spiked with $4 \times 10^6$ cells of CHA0 wild type, or its tpsA2 or tpsA4 deletion mutants. Thirty-two 2nd instar larvae were used per bacterial strain.

**d** Survival of *G. mellonella* larvae after injection of $2 \times 10^3$ cells into the hemocoel. Eighteen 7th instar larvae were used per bacterial strain. One experiment of each is shown and two more *P. xylostella* feeding and one *G. mellonella* injection are shown in Supplementary Fig. S5. Asterisks indicate significant differences of mutants to the wildtype (log-rank test, $p$ value < 0.05).
Further shows that the maximal expression of the bacteria colonize the insect gut (Fig. 4). The hemolymph but that the upregulation is initiated when compared to the hemo-
lymph when compared to the hemolymph mimicking Grace’s Medium (Supplementary Fig. S6 and Table S6). This may be related to defenses against insect immune responses. Furthermore, the bacteria are challenged by iron deprivation forcing a strong upregulation of pyoverdine synthesis and heme-acquisition related genes (Fig. 4 and Supplementary Table S6). Pathogenic bacteria need siderophores such as pyoverdine and heme-acquisition systems to acquire the essential iron from iron-binding proteins e.g., ferritin or transferrin in the gut lumen, the hemolymph, and the fat body. Also, the hemolymph pH is not acidic enough for the scarce free iron to be bioavailable for the bacteria [87–89]. However, mutants defective in pyoverdine production still show insecticidal activity [29], probably because the loss of pyoverdine is compensated by the production of other siderophores e.g., enantio-pyochelin during insect colonization [90, 91].

The Fit insect toxin substantially contributes to insect killing in systemic infections by CHA0 and other P. protegens/P. chlororaphis [15, 17, 20]. It possibly interferes with the activity of hemocytes as was shown for the related apoptotic toxin Mcf of P. luminescens [92, 93]. Previously, Fit was shown to be only produced in the insect hemolymph but not on plant roots using a mCherry-labeled FitD [20]. We can show now that fitD was among the 20 most upregulated genes in the G. mellonella-wheat comparison (Fig. 4 and Supplementary Table S6). Our study further shows that the maximal expression of fitD occurs in the hemolymph but that the upregulation is initiated when the bacteria colonize the insect gut (Fig. 4).

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

We compared the full protein sequence of CHA0 and 96 pseudomonads using ortholog analyses (Supplementary Fig. S7). We analyzed phylogenetic groups harboring strains showing plant beneficial interactions as well as plant-, insect-, and human pathogenic abilities (Supplementary Table S2). P. protegens/P. chlororaphis subgroups possess a set of specific traits absent in other Pseudomonas groups (Fig. 6 and Supplementary Fig. S7). We investigated whether CHA0 genes responding to lifestyle changes were common to other pathogenic and beneficial Pseudomonas. We focussed on genes with host or insect compartment specific expression that have emerged from this study and/or have been shown to contribute to insecticidal activity in earlier studies [16, 28] i.e., specific exoenzymes, exopolysaccharides, T6SS modules, and toxins.

Some genes with lifestyle specific expression patterns are distinct of the P. protegens subgroup including the transposase PPRCHA0.1961, Vgr1a elements e.g., the Rhsl effector [28] and TpsB1 (Fig. 6). This suggests that these genes were recently acquired by P. protegens or were lost in all other groups. We propose that some of these genes are specific for insect interactions. However, most functions in insecticidal activity are shared by the P. protegens and P. chlororaphis subgroups. Intriguingly, some of these traits are also present in the phylogenetically distant P. aeruginosa group [1, 2] harboring animal and human pathogens. Among these functions, we found a chitinase, the phospholipase PlcN, proteins related to the production of the exopolysaccharide Pel, T6SS components of the Vgr1A and Vgr1B modules, the Reb toxins or some proteins related to stress-response. The presence of these insect interaction-related CHA0 traits in very distant species such as P. aeruginosa (Fig. 6) suggests that either these are ancestral functions and were lost repeatedly during the evolution of Pseudomonas or that they have been independently acquired. Interestingly, the entomopathogen P. entomophila [24] lacks several of the CHA0 functions shared with the aeruginosa group including Pel, PsI, the Vgr1a, and b elements [28], and some stress-related proteins (Fig. 6). P. chlororaphis and P. protegens with the ability to colonize plant and insect environments seem to have a very distinct toolbox when compared to the rest of the analyzed species (Fig. 6 and Supplementary Fig. S7).

**Conclusions**

P. protegens and P. chlororaphis are bacterial species with multifaceted lifestyles as they can easily switch between plant and insect hosts. Our analyses of the P. protegens CHA0 transcriptomes across plant, insect and specific culture medium conditions significantly enhance our understanding of the shared and specific functions deployed across host-associated lifestyles. We have also shown how different functions are modulated over the course of an insect infection. Our results show that CHA0 deploys distinct toolsets to colonize plant roots, the insect gut, and the insect hemocoel with specific
expression in some environments (e.g., flagella on roots or the Fit toxin in the insect hemocoel). In contrast, we also discovered that antimicrobial metabolites, the T6SS, and exopolysaccharides serve as weapons or colonization factors across multiple environments. We also identified potential new factors involved in insect interactions of P. protegens CHA0 i.e., PPK and the transposase PPRCHA0_1961 and we demonstrated the contribution of TPS systems in insect pathogenicity. Based on the results presented here and our previous studies on insecticidal traits, we propose a comprehensive insect colonization and pathogenesis model for P. protegens CHA0 as summarized in Fig. 7.

We finally show that some key insect pathogenicity factors are conserved across Pseudomonas groups, while other factors are patchily distributed in P. protegens or P. protegens chlororaphis suggesting distinct evolutionary origins.
Data availability

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

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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