Plant Soil
https://doi.org/10.1007/s11104-024-07200-w

RESEARCH ARTICLE

A putative novel type of tight adherence (*tad*) like gene cluster of *Pseudomonas chlororaphis* PCL1606 exhibits a crucial role in avocado roots colonization, fostering its biological control activity

Blanca Ruiz-Muñoz · María Rodríguez-García · Zaira Heredia-Ponce · Sandra Tienda · Rafael Villar-Moreno · Eva Arrebola · A. de Vicente · Francisco M. Cazorla · José A. Gutiérrez-Barranquero

Received: 27 March 2024 / Accepted: 26 December 2024

Abstract

Aims *Pseudomonas chlororaphis* PCL1606 (PcPCL1606), displays strong antagonistic and biological control abilities against several soil-borne fungal pathogens mainly due to the production of the antifungal molecule 2-hexyl, 5-propyl resorcinol (HPR). HPR governs other beneficial phenotypes, suggesting its additional regulatory activity. Published transcriptomic data identifying HPR-regulated genes involved in the interaction of PcPCL1606 with the avocado rhizosphere were used as a target database to identify putative genes involved in avocado roots colonization.

Methods The induction of several consecutive genes that showed homology with genes encoding a putative type IV Flp/Tad (tight adherence) pilus but with a few differences from the Tad type A and B was observed. To study the role of this *tad*-like gene cluster in the biology of PcPCL1606, a chromosomal deletion mutant was constructed. The molecular characterization of the *tad*-like gene cluster and different in vitro and in vivo phenotypes related to colonization were addressed in the mutant strain respect to PcPCL1606.

Results The *tad*-like gene cluster was composed of five independent transcriptional units. Furthermore, the *tad*-like deletion mutant was impaired in early attachment, early biofilm formation, bacterial autoggregation and in root competitiveness in avocado plants and biocontrol activity against *R. necatrix*.

Conclusions These results expand our understanding about the role of HPR as a putative signalling molecule. This study revealed the importance of a putative novel type of a Tad system of PcPCL1606 in the avocado roots colonization, confirming that initial attachment to roots is a fundamental mechanism for the PcPCL1606 rhizospheric performance.

Keywords *Pseudomonas chlororaphis* PCL1606 · Avocado roots colonization · HPR signaling network · Tad-like pilus

---

Responsible Editor: Katharina Pawlowski.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-024-07200-w.

B. Ruiz-Muñoz · M. Rodríguez-García · Z. Heredia-Ponce · S. Tienda · R. Villar-Moreno · E. Arrebola · A. de Vicente · F. M. Cazorla · J. A. Gutiérrez-Barranquero (✉)
Department of Microbiology, Faculty of Sciences, University of Málaga, Málaga, Spain
e-mail: jagutierrez@uma.es

B. Ruiz-Muñoz · M. Rodríguez-García · Z. Heredia-Ponce · S. Tienda · R. Villar-Moreno · E. Arrebola · F. M. Cazorla · J. A. Gutiérrez-Barranquero
Department of Microbiology and Plant Protection, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", IHSM-UMA-CSIC, Málaga, Spain

M. Rodríguez-García · Z. Heredia-Ponce
Department of Plant and Microbial Biology, University of Zürich, 8008 Zurich, Switzerland

Introduction

The rhizosphere is the portion of soil intimately associated with plant roots and is directly influenced by the secretion of root exudates, which serve as a rich source of carbon and nitrogen, enhancing the flourishing microbial population in the vicinity of roots (Koprivova and Kopriva 2022). This fact makes the rhizosphere a competitive dynamic environment where beneficial, mutualistic, symbiotic, or pathogenic relationships between roots and soil microbes thrive. Thus, the rhizosphere provides a nutrient-rich environment that selectively recruits microorganisms from the surrounding soil, and some of these microorganisms show beneficial effects on plant health (Chepsergon and Moleleki 2023; Yuan et al. 2018). The initial attachment to plant roots constitutes the first physical step in the colonization process in many plant–microbe interactions and after the first weak-reversible primary attachment of bacteria, a second irreversible-phase attachment can occur, which is stronger and more specific (Wheatley and Poole 2018). In particular, some beneficial bacteria selectively recruited by plant root exudates should carry out efficient root colonization to exert beneficial effects on plants, such as plant growth promotion (Lopes et al. 2021; Lugtenberg and Kamilova 2009) and/or protection against pathogens via different mechanisms (Hacquard et al. 2017; Kwak et al. 2018; Yin et al. 2021). Thus, bacterial colonization of the rhizosphere has been described as a crucial mechanism for the establishment of beneficial plant-bacteria interactions, representing a key determinant of plant health and productivity (Knights et al. 2021; Liu et al. 2024). However, the molecular mechanisms underlying this complex interaction remain unclear. Among the different genetic features that can act during bacterial surface colonization, the tight adherence locus (*tad* locus) (also called *flp*/*tad* gene cluster), a subclass of the type IV secretion system that is common across bacteria and archaea (Giltner et al. 2012; Tomich et al. 2007), has been described for its importance in adhesion, biofilm formation and virulence mainly in animal and plant pathogenic bacteria (Andrade and Wang 2019; De Bentzmann et al. 2006; Nykyri et al. 2013; Schreiner et al. 2003). However, very little is known about the role of the *tad* gene cluster in rhizospheric beneficial bacteria, but recent reports suggest that it plays a key role in plant colonization by forming part of the extracellular matrix components (EMC) (Blanco-Romero et al. 2024; Cole et al. 2017).

Among the most important beneficial rhizospheric bacteria, members of the genus *Pseudomonas*, and specifically, those within the *Pseudomonas fluorescens* complex, have been one of the most widely studied for their great metabolic repertoire exhibiting a remarkable capacity to inhibit the growth of a wide spectrum of plant pathogens, employing strategies such as antibiosis, induction of plant defence mechanisms, and outcompeting pathogens for resources (Raaijmakers et al. 2009; Silby et al. 2011). Concomitantly, they directly contribute to plant development and health by facilitating nutrient availability, nitrogen fixation, modulation of plant hormones, and improving tolerance to environmental stresses (Sarraf et al. 2023; Solomon et al. 2024). Recently, the species *Pseudomonas chlororaphis* has attracted attention from an agricultural point of view for its great potential as a biological control agent (Arrebola et al. 2019; Liu et al. 2022, 2023; Wang et al. 2024; Wei et al. 2024) and for its genome plasticity and adaptation mechanisms (Loper et al. 2012; Molina-Santiago et al. 2021; Nikolaidis et al. 2020; Wang et al. 2015). *Pseudomonas chlororaphis* PCL1606 (PcPCL1606) is a model biocontrol rhizobacterium isolated from the rhizosphere of healthy avocado plants in an area affected by the soil-borne phytopathogenic fungus *Rosellinia necatrix* (Cazorla et al. 2006). PcPCL1606 produces the antifungal compound 2-hexil, 5-propyl resorcinol (HPR), which belongs to the “dialkylresorcinols" (DARs) family of compounds which have been recognized as novel signalling molecules (Brameyer et al. 2015). HPR compound has been directly linked to the antagonistic and biocontrol activities of PcPCL1606 (Calderón et al. 2013), and additionally it has been described to be crucial for root colonization, biofilm formation and survival (Calderón et al. 2014, 2019; Tienda et al. 2024).

Colonization of the rhizosphere by beneficial bacteria is an essential step towards improving plant performance (Knights et al. 2021; Liu et al. 2024) Remarkably, the colonization, biofilm formation and biological control activity of PcPCL1606 are under the control of the HPR compound, as it was stated above. Henceforth, to obtain deeper insight into the role of HPR in the beneficial interaction of PcPCL1606 with the avocado rhizosphere, transcriptomic data obtained in Tienda et al. (2024) were used in this study as a target database to identify putative candidate genes that could play a relevant role in avocado root colonization. Then, a *tad*-like gene cluster was detected to be overexpressed in PcPCL1606 during its interaction with the avocado roots, suggesting its important role in the initial attachment process. In addition, two types of Tad systems, type A and B, have been found associated in different *Pseudomonas* species, including some groups of *P. fluorescens* complex. In particular, both types (type A and B) are present in *P. chlororaphis* and *P. mandelii* groups (Blanco-Romero et al. 2020). Thus, the main aim of this study was to disentangle the role of the *tad*-like gene cluster in the colonization process of avocado roots by PcPCL1606. This study sheds light on the genetic and biological underpinnings that are involved in efficient bacterial colonization of the rhizosphere, thus promoting the potential benefits of these bacteria to plants.

Material and methods

Bacterial strains and culture conditions

The bacterial and fungal strains and plasmids used in this study are summarized in Table 1. Tryptone-peptone-glycerol (TPG; (Calderón et al. 2013)) medium was used for standard maintenance of wild-type PcPCL1606 and its derivatives at 25 °C. Lysogenic broth (LB) medium was used for culturing *Escherichia coli* DH5α and *Pseudomonas aeruginosa* PAO1 strains at 37 °C. King´s B (KB) agar medium was used for twitching motility. The bacterial strains were stored at −80 °C in LB broth supplemented with 20% glycerol. The media were supplemented with the appropriate antibiotics when required (kanamycin 50 µg/ml, gentamicin 80 µg/ml and rifampicin 10 µg/ml).

Table 1 Bacterial and fungal strains, and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference or source
Bacterial strains		
Escherichia coli DH5α	Host for the mutation plasmids; Km^{R} [a]	Boyer and Roulland-Dussoix (1969)
E. coli DH5α pGEM-T	Kanamycin resistance cassette donor strain; Km^{R}	Matas et al. (2014)
E. coli DH5α pRL662::*gfp2*	*E. coli* DH5α carrying the GFP fluorescent marker, Gm^{R} [b]	Bernal et al. (2017)
Pseudomonas chlororaphis PCL1606 (PcPCL1606)	Wild type strain, isolated from avocado rhizosphere	Cazorla et al. (2006)
PcPCL1606-Rif^{r}	Spontaneous mutant of PcPCL1606, Rif^{R} [c]	González-Sánchez et al. (2010)
PcPCL1606 wild type mCherry	mCherry-tagged PcPCL1606 wild-type, mini-Tn7 PA1/04/03mCherry; Gm^{R}	Heredia-Ponce et al. (2021)
PcPCL1606 Δ*pilA*	PcPCL1606 deletion mutant (*pilA* gene); Km^{R}	This study
PcPCL1606 Δ*pslE*	PcPCL1606 deletion mutant (*pslE* gene); Km^{R}	Heredia-Ponce et al. (2021)
Pseudomonas aeruginosa PAO1	Wild type strain	Stover et al. (2000)
Fungal strain		
Rosellinia necatrix CH-53	Wild type, isolated from avocado trees with symptoms of white root rot; high virulence	Pliego et al. (2008)
Plasmids		
pGEM-T *easy vector*	Cloning vector; 3016 bp; Amp^{R} [d]	Promega, Madinson, WI
pGEM-TDpil	*pilA* gene cloned into pGEM-T used for mutation on strain PcPCL1606. 3357 bp; Km^{R}; Amp^{R}	This study

[a] Km^{R}: kamanycin resistance

[b] Gm^{R}: gentamicin resistance

[c] Rif^{R}: rifampicing resistance

[d] Amp^{R}: ampicillin resistance

Selection and identification of putative HPR-regulated genes involved in bacterial colonization

In this study a RNAseq experiment previously performed by our research group (https://doi.org/10.6084/m9.figshare.23660028; Tienda et al. 2024) was used as a target database. We aimed to identify genes overexpressed in wild-type PcPCL1606 during its interaction with the avocado rhizosphere in comparison to the PcPCL1606 Δ*darB* mutant which is defective in HPR production. Genes with expression values greater than $\log_2$ (fold change) > 1 were considered to be differentially expressed. Nucleotide sequence searches were performed using nucleotide BLAST and BLASTx tools from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). In addition, protein functional annotation was carried out using eggNOG-mapper v2 (Cantalapiedra et al. 2021). A cluster of several overexpressed consecutive genes was further selected as a putative *tad*-like gene cluster involved in bacterial colonization.

Bioinformatic search to determine de number and types of Tad systems in PcPCL1606

The genome sequence of PcPCL1606 deposited at NCBI (Genbank: CP011110.1) was used to perform a bioinformatic search using the genome annotation to determine the number and types of Tad systems present in PcPCL1606. Subsequently, all the identified genes and proteins were identified by using blastX and Protein BLAST tools from NCBI, respectively.

Gene organization of a putative *tad*-like cluster

DNA and RNA were isolated from PcPCL1606 grown for 24 h at 25 ºC in TPG broth medium. Genomic PcPCL1606 DNA was isolated using a DNeasy UltraClean Microbial Kit (Qiagen). RNA was isolated using NucleoSpin® RNA Plant kit (MACHERY-NAGEL). Then, an additional step using a Turbo DNase kit (Invitrogen, Thermo Fisher Scientific) was used to remove DNA contamination from the RNA.. The RNA concentration was determined by a NanoDrop One/One^C UV–Vis spectrophotometer (Thermo Fisher Scientific) and RNA integrity was checked by 1% agarose gel electrophoresis. cDNA was obtained by using RNA as a substrate through the SuperScript III Reverse Transcriptase kit (Invitrogen) with 5 µg of total RNA in a final reaction volume of 20 µl. The procedure is divided into two different steps. First at all, a denature step was carried out on by incubation of 1 µl of random primers (75 mM), 1 µl dNTPmix (10 mM) and 5 µg RNA at 65 °C for 5 min, and subsequently incubated at 4 °C for 1 min. The second step consisting of the cDNA synthesis process was performed by the addition of cDNA synthesis mix (1 µl of 0.1 M of DTT, 1 µl of RNase OUT recombinant RNAse inhibitor, 1 µl of SuperScript III Reverse Transcriptase and 4 µl of 5X-First-Strand-Buffer) following incubation at 25 °C for 10 min. After that, a step at 50 °C for 50 min was carried out. The primers used for RT-PCR are listed in Table S2 and were designed with Primer3 software using the PcPCL1606 genome as a template (GenBank accession number: JQ663992). Then, a standard PCR amplification protocol was performed with all the primers listed in Table S2 using DNA and cDNA as templates. A control for DNA contamination was included in the amplification reaction.

Construction of the Δ*pilA* mutant

A PcPCL1606 deletion mutant of the two putative *pilA* genes located in the *tad*-like gene cluster, was constructed using the pGEM-T Easy Vector®. First, DNA fragments of approximately 1 kb, corresponding to the 5′ and 3′ flanking regions of the target gene, were amplified and fused using specific primers that included a BamHI site and a T7 primer sequence (Zumaquero et al. 2010; Heredia-Ponce et al. 2021) (Table S1). The resulting product was TA-cloned and inserted into pGEM-T and fully sequenced to discard possible mutations. The resulting plasmid was tagged with the *nptII* Km resistance gene obtained from pGEM-T-KmFRT-BamHI. For marker-exchange mutagenesis, the pGEM-T-Δgene-Km plasmid was introduced into the PcPCL1606 strain by electroporation using a Gene Pulser Xcell System (Bio-Rad) according to the manufacturer's instructions. The electrocompetent cells of PcPCL1606 were obtained according to (Choi et al. 2006). PcPCL1606 transformants were selected on TPG agar plates supplemented with kanamycin. To verify the deletion of the two consecutive *pilA* genes, each transform was subjected to PCR using specific primers to amplify a fragment within the *pilA* gene (Table S1). Growth curves of PcPCL1606 and the Δ*pilA* deletion mutant in TPG broth medium were performed.

In vitro early attachment and early biofilm formation assays

Early attachment and early biofilm formation assays were performed with the wild-type strain PcPCL1606 and the deletion Δ*pilA* mutant. These assays were performed on 24-well plates as previously described (Heredia-Ponce et al. 2021) with minor modifications. A mutant of PcPCL1606 in the *psl*-like polysaccharide gene cluster (Δ*pslE* mutant), obtained previously (Heredia-Ponce et al. 2021) was included as a negative control. Overnight cultures of the bacterial strains in TPG broth medium (150 rpm, 12 h at 25 °C) were used to adjust the bacterial inoculum at optical density (OD) values of 0.25 and 0.05 at a wavelength of 600 nm with sterile TPG broth medium for early attachment and early biofilm formation, respectively. A total of 900 µL of bacterial suspension was added to each well. After inoculation, the plates were incubated statically for 4 h for early attachment and 24 h for biofilm formation, both at 25 ºC. After that time, the culture of each well was carefully removed, and a distilled water wash step was performed. After that, 1.0 ml of 0.1% crystal violet (CV) solution was added to each well and incubated for 15 min. Then, the CV was removed carefully by several washing steps with distilled water to remove the remaining CV. The plates were dried at 37 °C for 2 h and then 1.2 ml of 96% ethanol was added to each well to solubilize the crystal violet. The amount of crystal violet present in each well represents the early attachment and early biofilm formation produced and was quantified by measuring the OD at a wavelength of 590 nm. Three independent experiments with at least four technical replicates per experiment and strain were performed.

Swarming, swimming, and twitching motility assays

To verify the motility differences between the wild-type strain and the Δ*pilA* deletion mutant, swimming, swarming, and twitching assays were conducted. Swimming and swarming motility assays were performed on TPG agar plates. Bacteria were stabbed using sterile toothpicks in 0.3% and 0.5% agar plates for swimming and swarming, respectively, and incubated at 25 °C. After 24 h and 48 h the motility areas were measured using Quantity One 1D Analysis Software (Bio-Rad). Three independent experiments and three technical replicates per experiment and strain were performed. Twitching motility assays were performed on KB 1% agar plates. Bacteria were stabbed using sterile toothpicks introducing them until the end of the plate and were incubated for 48 h at 25 °C for PcPLC1606 and Δ*pilA* mutant strains and 37 °C for *P. aeruginosa* PAO1 strain. After that time, 1 ml of 1% crystal violet solution was added to each plate which were incubated with movement for 15 min and immediately washed with water. Then, images were taken and compared. Three independent experiments and three technical replicates per experiment and strain were performed.

Antagonistic activity against *Rosellinia necatrix*

The antagonistic activity of the PcPCL1606 strain and the Δ*pilA* mutant was tested against the fungus *R. necatrix*, the causal agent of the avocado white root rot disease, as previously described (Calderón et al. 2013; Cazorla et al. 2006). To screen for in vitro antifungal activity, the *R. necatrix* CH53 strain was used, and the assay was performed on TPG plates as follows: a 0.6-cm-diameter mycelium disk from a 5-day-old fungus grown on PDA at 25 ºC was placed in the centre of a Petri dish of TPG agar medium. Then, the bacterial strains were inoculated approximately 2–3 cm from the fungus. Antagonistic activity was indicated by the inhibition of mycelial growth after 5 days of incubation at 25 ºC. Three independent experiments were performed. TPG agar plates with *R. necatrix* CH-53 without bacterial inoculation were included as positive control of fungal growth.

Bacterial sedimentation and aggregation assays

A sedimentation assay (Vo et al. 2022) was used to quantify the self-aggregation of the wild-type strain and its derivative Δ*pilA* mutant. Single colonies of the wild-type strain and the Δ*pilA* mutant grown on TPG agar plates were inoculated into tubes with 5 ml of TPG broth and incubated overnight at 180 rpm at 25 °C. Then, cells were harvested by centrifugation at a low speed (6000 rpm for 3 min) to prevent cell lysis and the pellets were washed twice with 0.85% sterile NaCl solution. Then, the bacterial cell suspensions were adjusted to an OD of 0.5 at 600 nm (approximately 10^8 cfu/ml) in M9 minimal medium supplemented with avocado rhizosphere exudates obtained following a protocol described previously (Polonio et al., 2017), in a 9:1 ratio (M9 medium:exudates) respectively. 3 ml of each adjusted culture was transferred to a thin glass capillary and left statically at 25 ºC. At 0, 4, 8, and 24 h, 100 µl aliquots were carefully taken from the top of the culture and the OD at a wavelength of 600 nm was measured. To validate the OD measurements, bacterial counts were performed on the collected samples at the mentioned time points. The assays were carried out in triplicate, with at least three technical replicates of each.

Scanning electron microscopy was performed to visualize the bacterial self-aggregation process in both the wild-type strain and the Δ*pilA* mutant following a protocol previously described with minor modifications (Zeriouh et al. 2014). At the 4-h time point of the sedimentation assay, 10 µl aliquots were carefully taken from the middle part of the bacterial cultures (PcPCL1606 and the Δ*pilA* mutant) and deposited onto polylysinated discs. Subsequently, the drops were allowed to dry at 37 °C for 24 h. The bacterial drops were then fixed in a solution of 0.1 mM sodium cacodylate (pH 7.2) and 2.5 mM glutaraldehyde for 24 h at room temperature. Bacteria were then dehydrated using an ethanol series ranging from 50 to 100% and dried using a Bal-Tec CPD 030 critical point dryer. Finally, the dried samples were coated with a thin layer of gold using a Leica EMSCD050 coater prior to visualization under a JEOL JSM-6490 LV microscope.

Root competitiveness

Bacterial competitive index (CI) experiments were performed on avocado plant roots using the wild-type strain PcPCL1606 (gentamicin resistant) and the Δ*pilA* mutant (kanamycin resistant). The avocado plant roots were disinfected in 0.1% sodium hypochlorite solution (20 min), washed in sterile water for 20 min, and immersed in the bacterial mixed cultures in 1:1 ratio (final concentration of 10^8 cfu/ml) for 20 min, as previously described (Calderón et al. 2014). The control plant roots were incubated with TPG broth medium as a reference. Inoculated plants were transplanted into pots filled with vermiculite and placed into a greenhouse at 25 °C and root samples were collected at 4, 8 and 24 h. For that, the plants were removed from the pots, and superficial root samples (1 g) were randomly collected with a scalpel and homogenized in 5 ml of NaCl solution (0.85%) for 3 min. Tenfold serial dilutions were plated on TPG agar plates supplemented with gentamicin for PcPCL1606 and kanamycin for the Δ*pilA* mutant. The competitiveness was expressed as a competitive index (CI) based on the number of cfu/g of fresh root weight. The CI was calculated by determining the ratio of each strain to the total population. The assay was independently performed three times with three replicates each.

Biocontrol assays

The biocontrol assays were performed as previously described (Cazorla et al. 2006). Commercial 6-months-old avocado plants (cv. Walter Hole) from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain) were used. The roots of these plants were disinfected with 0.1% sodium hypochlorite solution for 20 min and washed in sterile water for 20 min. For the inoculation of bacterial strains, avocado roots were introduced in bacterial suspension culture (10^8 cfu/ml) or in a sterile TPG broth medium as the control treatment for 20 additional minutes (Calderón et al. 2013). Then, the avocado plants were transplanted into pots containing potting soil and subsequently infected with *R. necatrix* by introducing 6 colonized wheat grains with *R. necatrix* per pot. The different treatments used included the following: the wild-type strain PcPCL1606, the Δ*pilA* deletion mutant, the *pslE* deletion mutant as a control that is impaired in its biocontrol ability (Heredia-Ponce et al. 2021), plants inoculated only with the fungal pathogen as a positive control of the disease, and an environmental control treatment in which plants were neither inoculated nor infected. Two independent experiments, each with 15 avocado seedlings per strain, were performed. Aerial symptoms of white root rot disease were recorded on a scale from 0 to 3 (from 0 – not affected to 3 – dead plant) as previously described. From the disease index curve, the area under the disease progress curve (AUDPC) was calculated in groups of 5 plants and statistically compared with each strain after all values were normalized to the wild-type average for each independent experiment.

Interbacterial competition assay

Bacterial in vitro competition experiments were performed using the spontaneous mutant PcPCL1606Rif^R, the Δ*pilA* mutant, and the *Escherichia coli DH5α* pRL662::*gfp2* (prey) strains (Bernal et al. 2017). Overnight cultures of *Pseudomonas* and *E. coli* strains were grown in LB broth media at 25 ºC and 37 ºC for *Pseudomonas* and *E. coli* strains, respectively. Then, the cultures were washed in 0.85% sterile sodium chloride solution and were adjusted to an OD of 1.0 at 600 nm. Each *Pseudomonas* strain was mixed with the *E. coli* strain at a 1:1 ratio. Tenfold serial dilutions were performed to quantify the colony forming units (cfu) per ml at the beginning of the experiment using antibiotic selection. Then, 10 µL drops were spotted onto LB agar plates and incubated at 28 °C for 5 h. The bacterial mass from each competition drop was recovered and resuspended in 1 mL of 0.85% sodium chloride sterile solution, serially diluted, and plated onto LB agar plates with antibiotic selection. The CI was calculated as follows: CI = ((final attacker count/final prey count)/(initial attacker count/initial prey count)). The CI value is expressed as the logarithm in base 10 of the index. Three biologically independent experiments were performed.

Results

A putative novel type of Tad system in PcPCL1606

The species *P. chlororaphis* contains two types of Tad systems called type A and B (Blanco-Romero et al. 2020). A bioinformatic search to determine the number and types of Tad systems in PcPCL1606 was performed. In PcPCL1606 there are two Tad systems (Fig. S1). One of them belonged to the type B similar to that present in *P. ogarae* (formerly *P. fluorescens*) F113. The other Tad system that was under the study in this work, showed a similar genetic structure to the type A Tad system of *P. aeruginosa*, with some rearrangements and some relevant differences. There were some missing genes in our Tad system in comparison with that of *P. aeruginosa* such as *rcpA* and *tadD*, but instead of having only one pilin gene like in *P. aeruginosa* it has two pilin genes like the type B Tad system present in F113. Therefore, this Tad system in PcPCL1606 resembles a hybrid between the type A and B described and for this reason it has been proposed that could be a putative novel type of Tad system. To avoid repeating "putative novel type of Tad system" throughout the complete manuscript, we will refer to the cluster simply as *tad*-like gene cluster.

Tad-like gene cluster is moderately activated depending on HPR production during the interaction with avocado roots

A transcriptomic approach recently published by our research group (Tienda et al. 2024), was used as a target database to identify putative candidate genes involved in the colonization of avocado roots which were under the control of HPR compound. Interestingly, from the 298 overexpressed genes (Fig. 1A), several consecutive genes predicted to be homologous to a putative *flp*/*tad*-like gene cluster were among the most overexpressed genes in the wild-type strain (Fig. 1B). The *tad* gene cluster has been shown to be involved in bacterial colonization of different hosts (O'Connell Motherway et al. 2011). A functional annotation of the predicted proteins from the genes belonging to the *tad*-like gene cluster of PcPCL1606 was carried out using eggNOG-mapper v2 (Cantalapiedra et al. 2021) and the results are summarized in Table S3. The PCL1606_53930, and PCL1606_53940 locus tags encode two consecutive pilin genes (called *pilA* genes).

The *tad*-like gene cluster is composed of five independent transcriptional units

To determine the transcription of the *tad*-like gene cluster, RT-PCR experiments were carried out using genomic DNA and mRNA from wild-type PcPCL1606 grown in TPG broth medium. RT-PCR analysis and gene orientation revealed that the transcription of these genes occurred in five independent transcriptional units which was confirmed by amplification of the connecting areas between the sequential ORFs and the internal regions of the transcripts (Fig. 2A and B). The amplification of genomic DNA was used as a control. Transcriptional unit 1 was composed of four genes, an autotransporter domain containing-protein (ADCP), a response regulator and two *pilA* genes. The second transcriptional unit was

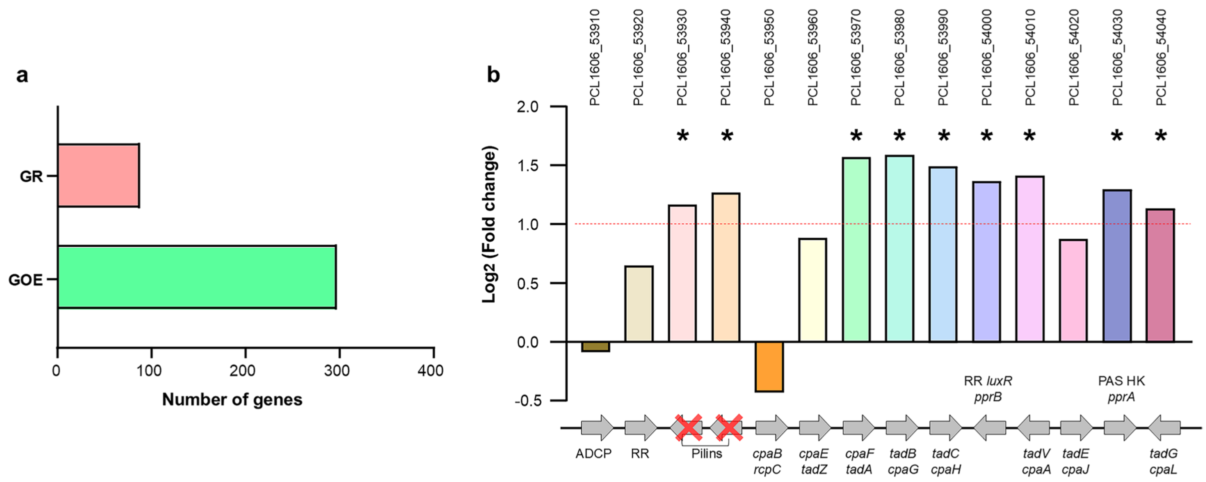

Fig. 1 Differentially expressed genes (DEGs) dependent on HPR production during the interaction of PcPCL1606 and the PcPCL1606 Δ*darB* mutant (impaired in HPR production) with avocado roots. **a** Bar chart showing genes repressed (GR, 88 genes) and genes overexpressed (GOE, 298 genes) in PcPCL1606 in the absence or presence of HPR. **b** Bar chart showing the differential expression of genes belonging to a homologous gene cluster to the type IVb *flp/tad* (tight adherence) gene cluster. Genes with a $\log_2$ fold change > 1 (HPR-overexpressing genes) are genes with higher expression than the red dotted line marked with an asterisk. The sizes of the different genes are not in proportional to their original sizes. Red crosses indicate genes that were mutated by deletion. The locus tag number of each gene is included in the upper part of the bar graph

composed of 5 genes, namely, *cpaB*, *cpaE*, *cpaF*, *tadB* and *tadC*. The third transcriptional unit was composed of two genes, a *luxR* response regulator, and a prepilin peptidase *tadV*. The fourth transcriptional unit was composed of of two genes, *tadE* and a PAS domain sensor histidine kinase. Finally, the fifth transcriptional unit was composed of at least of two genes *tadG* and a hypothetical protein. Thus, these results indicated that transcription of these 15 genes was done in five independent polycistronic mRNAs.

The *tad*-like gene cluster is involved in early attachment and early biofilm formation on abiotic surfaces, but not in swimming, swarming, antagonistic activity or interbacterial competition

A clean deletion mutant that included both *pilA* genes was constructed in this study to test the functionality of the *tad*-like gene cluster. These two small consecutive genes were selected because they generate the main proteins that form the structure of the pili (Giltner et al. 2012). To investigate the role of the *tad*-like gene cluster in early bacterial attachment at 4 h, short-term attachment experiments on polystyrene microtiter wells plates and crystal violet staining were carried out to compare the wild-type strain PcPCL1606 and the Δ*pilA* deletion mutant strains. The results revealed that the PcPCL1606 *tad*-like mutant (Δ*pilA*) showed a nearly 40% reduction in surface attachment (Fig. 3a). Similarly, with respect to early biofilm formation at 24 h, similar results to the initial attachment were observed. The Δ*pilA* mutant was impaired in early biofilm formation, showing a reduction of up to 35% in comparison to the biofilm formation values of PcPCL1606 (Fig. 3b). In both assays, we included the PcPCL1606 *psl*-like mutant (Δ*pslE*), which has been previously described to be involved in early attachment and biofilm formation (Heredia-Ponce et al. 2021), as a control for attachment deficiency. The Δ*pilA* mutant did not show any difference in swimming, swarming, antagonistic activity against *R. necatrix*, or interbacterial competition against *E. coli* with respect to the wild-type strain PcPCL1606. PcPCL1606 did not show twitching motility (Fig. S2).

The *tad*-like gene cluster is essential for cell-to-cell aggregation

To decipher the possible role of the *tad*-like gene cluster in bacterial autoaggregation, we performed

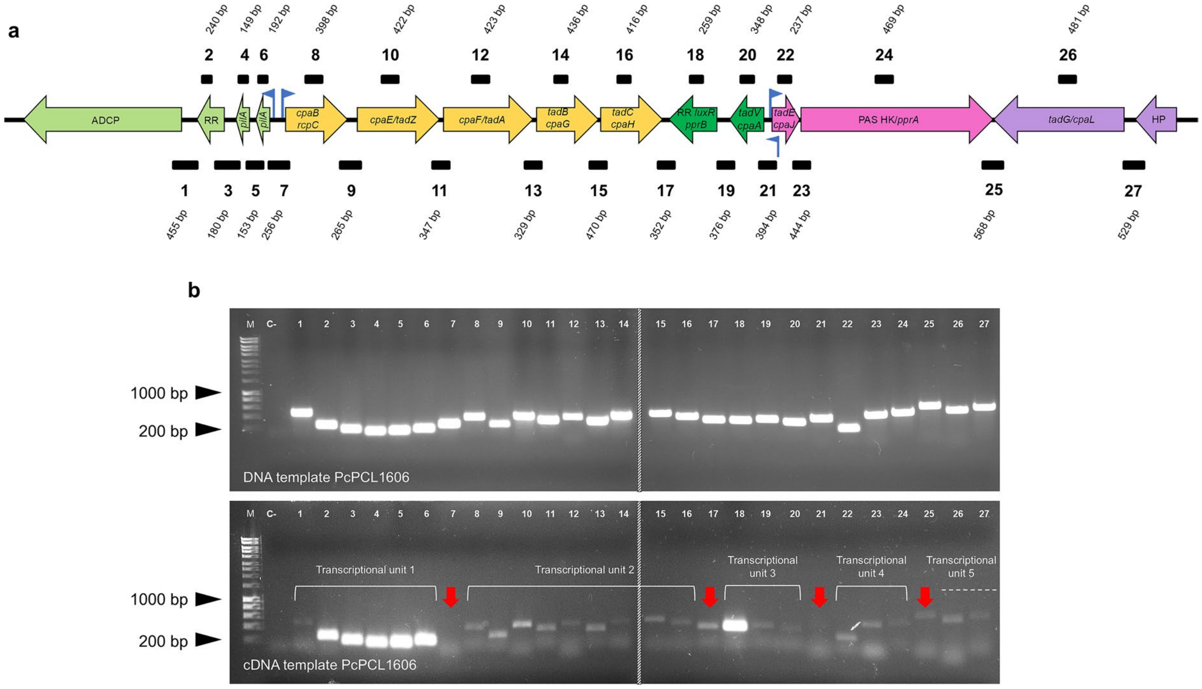

Fig. 2 RT−PCR analysis of the tad-like gene cluster. **a** Schematic representation of the tad-like gene cluster and the amplified region obtained during the RT−PCR experiments. Blue arrows correspond with prediction of promoter sequences using BPROM tool. **b** PCR products from RT−PCR experiments using genomic DNA and cDNA as templates obtained from the wild-type strain PcPCL1606 after 16 h of incubation at 25 °C on TPG broth medium. The primer pairs used are detailed in Supplementary Table 2. Lane M, molecular weight marker; Lane C-, negative control. ADCP: autotransporter domain-containing protein; RR: response regulator; HP: hypothetical protein

a sedimentation assay that measured the settling kinetics of standing cultures over time. For that, PcPCL1606 and the Δ*pilA* mutant were incubated statically and time-dependent variations in the optical density (OD) values of the upper part of the cultures were recorded. The results showed clear differences in the aggregation kinetics, while cells of the wild-type strain sedimented promptly, cells of the Δ*pilA* mutant displayed a slower sedimentation profile, with higher OD values (Fig. 4A). Moreover, to corroborate the OD measurements bacterial counts were performed on the same collected samples. Consistently, the number of PcPCL1606 cells (log cfu/ml) at the top of the culture was lower than that of to the Δ*pilA* mutant after 4 and 8 h of incubation (Fig. 4B). However, after 24 h, there was no significant difference in the bacterial counts. Given that the most marked difference in the autoaggregation process occurred after 4 h of incubation, this phenomenon was analysed by scanning electron microscopy (SEM). SEM micrographs strongly demonstrated that the autoaggregation phenotype of the Δ*pilA* mutant was impaired in auto-aggregation phenotype, as the cells were less aggregated than those of the wild-type strain, and in the form of microcolonies (Fig. 4C).

The *tad*-like gene cluster is required for successful root competitiveness and for the biocontrol activity of PcPCL1606

To explore the potential involvement of the tad-like gene cluster in the avocado root competitiveness of PcPCL1606, bacterial competitive index experiments were performed using the wild-type strain PcPCL1606 and the Δ*pilA* mutant (Fig. 5A). The results showed that the Δ*pilA* mutant was less competitive than PcPCL1606 in the avocado roots displaying lower CI values at 4 h (0.68 for PcPCL1606 and 0.32 for the Δ*pilA* mutant), 8 h (0.85 for PcPCL1606 and 0.15 for the Δ*pilA* mutant) and 24 h (0.78 for PcPCL1606 and 0.22 for the Δ*pilA* mutant)

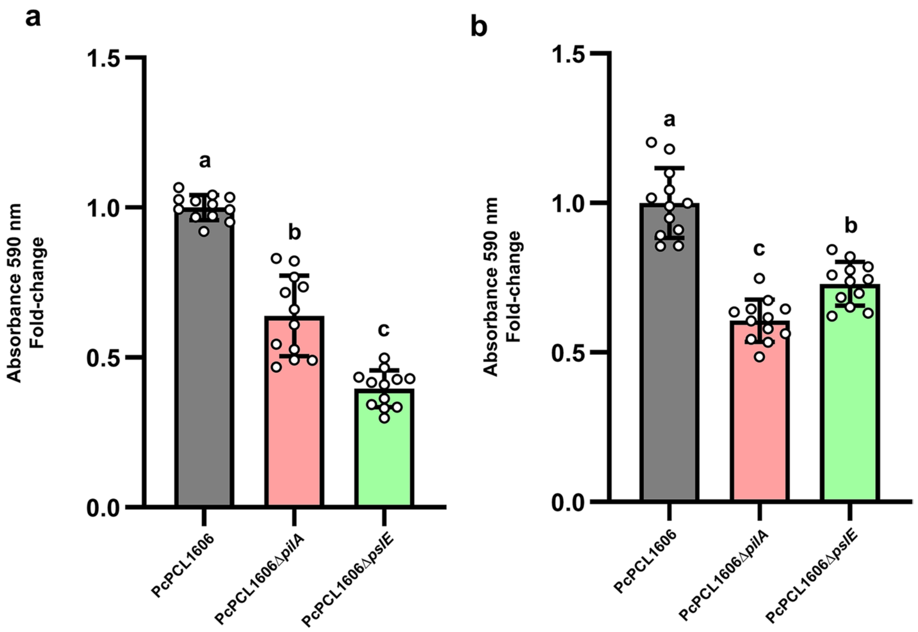

Fig. 3 Early attachment and biofilm formation assays to abiotic surfaces. **a** The initial attachment (4 h) and (**b**) biofilm formation (24 h) to abiotic surfaces were assessed using a crystal violet (CV) staining assay and the absorbance at 590 nm was measured. The results are displayed as the CV absorbance in the form of a fold-change relative to the wild-type average. The *P. chlororaphis* PCL1606 wild-type strain (PcPCL1606), PcPCL1606 *tad*-like mutant (Δ*pilA*) and PcPCL1606 Psl-like mutant (Δ*pslE*) strains were tested. Four technical replicates and three independent experiments were performed in both independent assays. Statistical analysis was performed using one-way ANOVA with post-hoc Bonferroni correction ($P \leq 0.05$). Different letters indicate significant differences. Error bars show the standard deviation (SD)

with statistically significant differences at all time points.

Finally, to determine whether the *tad*-like gene cluster might also have an influence on the protection of plants against soil-borne plant pathogenic fungi, biocontrol assays against avocado white root rot disease caused by *R. necatrix* were performed. Aerial symptoms were evaluated using the previously described disease scale (Cazorla et al. 2006). The area under the disease progression curve (AUDPC) revealed that when the avocado plants were inoculated with PcPCL1606, the disease index was reduced by 50% in comparison with that of the control plants without bacterial inoculation (Fig. 5B). Alternatively, when the plants were inoculated with the Δ*pilA* mutant, disease development appeared to be similar to that observed in previously uninoculated avocado plants, with no significant differences between the two groups (Fig. 5B). These results indicate a lack of biological control activity of either, the Δ*pilA* mutant, or the Δ*pslE* mutant, which was included as a negative control since it was previously described as lacking in biocontrol activity (Heredia-Ponce et al. 2021).

Discussion

The rhizosphere, the plant root-soil interface, is home to a plethora of microorganisms many of which are beneficial to plants (Chepsergon and Moleleki 2023; Liu et al. 2024). Notably, rhizosphere bacteria have a significant influence on the physiology and development of plants, and beneficial rhizobacteria play a pivotal role in plant nutrition, plant growth promotion, and plant disease interactions (Mendes et al. 2013). *Pseudomonas chlororaphis* PcPCL1606 is a beneficial model rhizobacterium isolated from avocado roots that displays antagonistic and biocontrol effects on different soil borne-fungal pathogens (Cazorla et al. 2006), mainly due to the production of the antifungal Compound 2-hexyl, 5-propil resorcinol (HPR; Calderón et al. 2013). The HPR compound in PcPCL1606 has been reported to additionally control

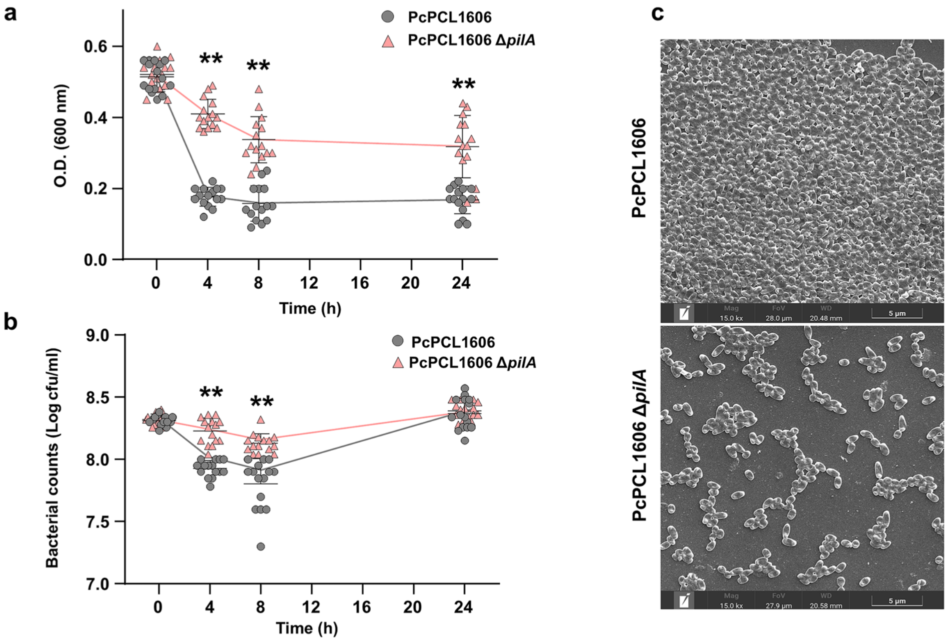

Fig. 4 Bacterial autoaggregation (cell–cell interaction). **a** Optical density values of the air–liquid interface in the cell suspensions under static conditions at 25 ºC after 4, 8 and 24 h. **b** Bacterial counts (log cfu/ml) of the air–liquid interface in the cell suspensions under static conditions at 25 ºC after 4, 8 and 24 h. ** indicates significant differences between the mutant and the wild-type strain according to unpaired Student's t test at $P \leq 0.005$. Error bars show the standard deviation (SD). **c**) Scanning electron micrographs of the air–liquid interface after 4 h of incubation. The scale bars are 5 µm

relevant biological processes such as colonization, persistence, and biofilm formation on avocado roots (Calderón et al. 2014, 2019). HPR belongs to dialkylresorcinols (DARs) family, a new class of signalling molecules (Brameyer et al. 2015). For beneficial bacteria to exert their plant-beneficial functions, successful root attachment and subsequent biofilm formation, which are the main steps in the rhizosphere colonization process, are essential (Knights et al. 2021; Wheatley and Poole 2018). Hence, rhizosphere colonization is considered a fundamental step for beneficial microbes to protect plants from pathogens and/or improve plant health (Santoyo et al. 2021; Zboralski and Filion 2020). This intricate process involves different molecular mechanisms (Knights et al. 2021) and occurs in a sequential manner, beginning with rhizosphere chemotaxis, followed by root attachment, and culminating in biofilm formation that facilitates the interaction of microorganisms with the host plant (Liu et al. 2024; Wheatley and Poole 2018).

In this study we focused on deciphering novel mechanisms involved in rhizosphere colonization under the umbrella of the HPR signalling network. Based on a recently published transcriptomic study (Tienda et al. 2024), of 298 genes overexpressed in the presence of HPR (Fig. 1A) we identified a putative type IVb *flp/tad*-like gene cluster in which most of the genes were overexpressed when HPR was produced during the interaction with avocado roots (Fig. 1B) suggesting the relevance of this *tad*-like gene cluster during the interaction of PcPCL1606 with avocado roots. The *tad*-like gene cluster is widespread in bacteria and archaea (Giltner et al. 2012; Tomich et al. 2007), and has been extensively studied in plant and animal pathogenic bacteria; this cluster plays an important role in adhesion, biofilm

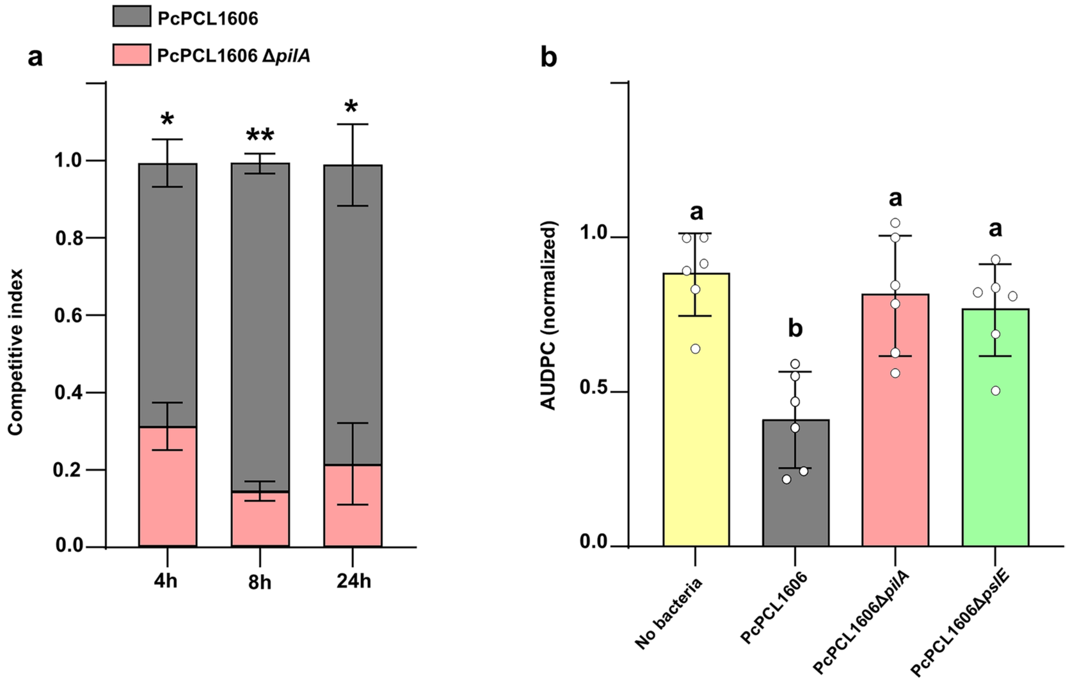

Fig. 5 In vivo experiments: root competitiveness and biocontrol activity. **a** Competitiveness assay on avocado roots. The *P. chlororaphis* PCL1606 wild-type strain (PcPCL1606) and PcPCL1606 *tad*-like mutant (Δ*pilA*) were coinoculated into the roots to analyse their competitiveness. The CI was calculated by determining the ratio of each strain to the total population at 4, 8 and 24 h of competition. * and ** indicate significant differences between the mutant and the wild-type strain according to unpaired Student's t test at $P \leq 0.05$ and $P \leq 0.005$, respectively. Error bars show the standard deviation (SD). **b** Biological control of *Rosellinia necatrix* in 6-month-old avocado plants. The area under the disease progression curve (AUDPC) is represented in this bar graph. Every point is the average value from 5 independent avocado plants. PcPCL1606, PcPCL1606 Δ*pilA* and PcPCL1606 Psl-like mutant (Δ*pslE*) were tested. Avocado plants used as a positive control for disease were inoculated with *R. necatrix* without added bacteria (no bacteria), and noninoculated plants were used as negative (nonrepresented). Statistical analysis was performed using one-way ANOVA with post hoc Bonferroni correction ($P \leq 0.05$). Different letters indicate significant differences. Error bars show the standard deviation (SD)

formation and virulence (Andrade and Wang 2019; De Bentzmann et al. 2006; Kachlany et al. 2000; Nykyri et al. 2013). However, the role of this *tad* gene cluster in beneficial rhizobacteria, particularly, those belonging to the genus *Pseudomonas*, is limited (Blanco-Romero et al. 2024; Cole et al. 2017). In silico analysis has shown that the genetic organization of the *tad* gene cluster is complex and diverse (Bernard et al. 2009; Blanco-Romero et al. 2020; Kachlany et al. 2001). Two types of Tad systems, type A and B, have been found *P. chlororaphis* (Blanco-Romero et al. 2020). In PcPCL1606 there are two Tad systems, one like the type B present in *P. ogarae* F113 and the other Tad system that was under the study in this work, showed a similar genetic structure to the type A Tad system of *P. aeruginosa*, with some rearrangements and some relevant differences an including features from the type B. Therefore, this Tad system in PcPCL1606 resembles a hybrid between the type A and B described, being proposed as a putative novel type of Tad system.

To determine whether the *tad*-like gene cluster present in PcPCL1606 has an operon structure, RT-PCR experiments were performed and revealed that the *tad*-like gene cluster of PcPCL1606 is organized into five transcriptional units (Fig. 2), different from what was previously observed in *tad* loci of *P. aeruginosa* which were organized into five transcriptional units (Bernard et al. 2009), or in *Yersinia enterocolitica* which all the genes of the *tad* gene cluster were transcribed as a single polycistronic mRNA forming an operon (Schilling et al. 2010).

To disentangle the function of the *tad-like* gene cluster in PcPCL1606, a deletion mutant was constructed that included the two *pilA* consecutive genes present in this gene cluster, which generates the main proteins that form the structure of the pili (Giltner et al. 2012). PcPCL1606 and the Δ*pilA* mutant were tested for early attachment and early biofilm formation (Fig. 3). In both cases, the Δ*pilA* mutant exhibited impaired early attachment (Fig. 3a) and early biofilm formation (Fig. 3b), compared with the wild-type PcPCL1606, which exhibited a decrease of approximately 40% in both phenotypes. In strains belonging to the *Pseudomonas fluorescens* complex, the *tad* gene cluster has been described to be involved in colonization and biofilm formation (Blanco-Romero et al. 2024; Cole et al. 2017; Lugtenberg et al. 2001), and the *tad* gene cluster is considered one of the main components of the extracellular matrix (Blanco-Romero et al. 2024). In addition, the *tad* gene cluster in *P. ogarae* F113, plays a crucial role in the adaptation to the rhizosphere since a mutation in the major *pilA* gene (*flp-1*) was shown to be involved in competitive colonization of the rhizosphere (Blanco-Romero et al. 2024).Similarly, for PcPCL1606, the competitive colonization of the rhizosphere by the Δ*pilA* mutant was impaired at three different time points in comparison with the wild-type PcPCL1606 (Fig. 5a), suggesting that the *tad*-like gene cluster plays a relevant role during the initial attachment to avocado roots. The rhizosphere is a highly competitive environment, where bacteria aggressively compete with other microbial inhabitants to survive and for niche colonization (Chepsergon and Moleleki 2023). Thus, PcPCL1606, must outcompete other microbes in the avocado rhizosphere. Different soil bacteria from the Myxococcales order (for instance *Bdellovibrio bacteriovorus* and *Myxococcus xanthus*) have developed a bacterial predator strategy to consume other microbes via different mechanisms of action (Laloux 2020; Thiery and Kaimer 2020). Specifically, in *M. xanthus*, a *tad* gene cluster has recently been shown to promote interbacterial competition through contact dependent killing (Seef et al. 2021) in combination with an atypical type 3-like protein secretion system (Thiery et al. 2022). Moreover, the type VI secretion system (T6SS) in PcPCL1606, has been shown to be activated upon contact with *Bacillus subtilis* cells, highlighting the importance of the T6SS in cell-to-cell interactions (Molina-Santiago et al. 2019). An interbacterial competition assay was carried out with PcPCL1606 or the Δ*pilA* mutant. However, the results confirmed that the *tad*-like gene cluster of PcPCL1606 was not necessary for interbacterial competition via contact-dependent killing (Fig. S2). Instead, this finding suggests that its primary role would be in attachment and biofilm formation to the avocado rhizosphere. Cell-to-cell interactions are among the earliest steps in the formation of bacterial biofilms (Sorroche et al. 2012). In this sense, bacterial autoaggregation, a phenomenon shown by many bacteria, refers to the ability of the bacteria to bind themselves, and is also related to surface attachment during the first steps in biofilm formation (Trunk et al. 2018). The *tad* gene cluster has been reported to be necessary for autoaggregation and biofilm formation in *Aggregatibacter actinomycetemcomitans* (Kachlany et al. 2000, 2001) and recently in *Vibrio vulnificus* (Pu and Rowe-Magnus 2018). The ability of PcPCL1606 to autoaggregate in vitro with respect to the Δ*pilA* mutant was tested, and interestingly the autoaggregation process was reduced in the mutant, which displayed a slower sedimentation profile (Fig. 4a, b). A discrepancy was observed at 24 h between the OD values and colony counts, which could be caused by the accumulation of byproducts of microbial metabolism. This phenotype was also confirmed by scanning electron microscopy at 4 h (Fig. 4c), where the cells of the Δ*pilA* mutant were less aggregated and were less abundant than those of the wild-type, appearing in the form of microcolonies. In some beneficial rhizobacteria, competitive colonization of roots is considered a relevant feature for exceptional biocontrol (Barahona et al. 2011; Montes-Osuna et al. 2021; Pliego et al. 2008; Tran et al. 2007). As the Δ*pilA* mutant of PcPCL1606 was affected in competitive root colonization and biofilm formation, its biocontrol ability against *R. necatrix* was tested in avocado plants. Interestingly, our results strongly confirmed that the *tad*-like gene cluster was involved in the biological control activity of PcPCL1606 (Fig. 5b). Competitive colonization of the rhizosphere improved biocontrol activity in *P. ogarae* F113 (Barahona et al. 2011), and the *tad* gene cluster of this biocontrol rhizobacterium has been linked to rhizosphere colonization (Blanco-Romero et al. 2024).

Thus, the results obtained in this work strongly suggest that in PcPCL1606, an efficient competitive rhizosphere colonization promoted by Tad-like pilus, could play a fundamental role in PcPCL1606 performance during the interaction with the avocado rhizosphere. Moreover, competitive colonization of the rhizosphere under the control of the HPR compound, provides new insights into the function of HPR as a putative signalling molecule.

Author contributions BRM: Main experimental work, data acquisition and analysis, Writing – original draft. MRG and ZHP: data acquisition and analysis at the initial stage of this work. ST and RVM: Investigation support. EA: Methodology, Funding acquisition. ADV Conceptualization. FMC: Conceptualization, Methodology, Funding acquisition, Supervision. JAGB: Conceptualization, Methodology, Supervision, Writing – review & editing.

Funding Funding for open access publishing: Universidad de Málaga/CBUA. This work was supported by PID2021-123713OB-I00 research project from the Ministerio de Ciencia, Innovacion y Universidades (Spain) and UMA18-FEDERJA-046 grant from Junta de Andalucía, both partially supported by the European Union (FEDER).

Data Availability Non applicable.

Declarations

Conflict of interest The authors declare there is no conflict of interest.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

References

Andrade M, Wang N (2019) The Tad pilus apparatus of '*Candidatus Liberibacter asiaticus*' and its regulation by VisNR. Mol Plant Microbe Interact 32:1175–1187. https://doi.org/10.1094/MPMI-02-19-0052-R

Arrebola E, Tienda S, Vida C, de Vicente A, Cazorla FM (2019) Fitness features nvolved in the biocontrol interaction of *Pseudomonas chlororaphis* with host plants: The case study of PcPCL1606. Front Microbiol 10:719. https://doi.org/10.3389/fmicb.2019.00719

Barahona E, Navazo A, Martínez-Granero F, Zea-Bonilla T, Pérez-Jiménez RM, Martín M, Rivilla R (2011) *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. Appl Environ Microbiol 77:5412–5419. https://doi.org/10.1128/AEM.00320-11

Bernal P, Allsopp LP, Filloux A, Llamas MA (2017) The *Pseudomonas putida* T6SS is a plant warden against phytopathogens. ISME J 11:972–987. https://doi.org/10.1038/ismej.2016.169

Bernard CS, Bordi C, Termine E, Filloux A, de Bentzmann S (2009) Organization and PprB-dependent control of the *Pseudomonas aeruginosa tad* Locus, involved in *flp* pilus biology. J Bacteriol 191:1961–1973. https://doi.org/10.1128/JB.01330-08

Blanco-Romero E, Garrido-Sanz D, Rivilla R, Redondo-Nieto M, Martín M (2020) In silico characterization and phylogenetic distribution of extracellular matrix components in the model rhizobacteria *Pseudomonas fluorescens* F113 and other Pseudomonads. Microorganisms 8:1740. https://doi.org/10.3390/microorganisms8111740

Blanco-Romero E, Garrido-Sanz D, Durán D, Rybtke M, Tolker-Nielsen T, Redondo-Nieto M, Rivilla R, Martín M (2024) Role of extracellular matrix components in biofilm formation and adaptation of *Pseudomonas ogarae* F113 to the rhizosphere environment. Front Microbiol 15:1341728. https://doi.org/10.3389/fmicb.2024.1341728

Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 14:459–472. https://doi.org/10.1016/0022-2836(69)90288-5

Brameyer S, Kresovic D, Bode HB, Heermann R (2015) Dialkylresorcinols as bacterial signaling molecules. Proc Natl Acad Sci USA 112:572–577. https://doi.org/10.1073/pnas.1417685112

Calderón CE, Pérez-García A, de Vicente A, Cazorla FM (2013) The *dar* genes of *Pseudomonas chlororaphis* PCL1606 are crucial for biocontrol activity via production of the antifungal compound 2-Hexyl, 5-Propyl Resorcinol. Mol Plant Microbe Interact 26:554–565. https://doi.org/10.1094/MPMI-01-13-0012-R

Calderón CE, de Vicente A, Cazorla FM (2014) Role of 2-hexyl, 5-propyl resorcinol production by *Pseudomonas chlororaphis* PCL1606 in the multitrophic interactions in the avocado rhizosphere during the biocontrol process. FEMS Microbiol Ecol 89:20–31. https://doi.org/10.1111/1574-6941.12319

Calderón CE, Tienda S, Heredia-Ponce Z, Arrebola E, Cárcamo-Oyarce G, Eberl L, Cazorla FM (2019) The compound 2-Hexyl, 5-Propyl resorcinol has a key role in biofilm formation by the biocontrol rhizobacterium *Pseudomonas chlororaphis* PCL1606. Front Microbiol 10:396. https://doi.org/10.3389/fmicb.2019.00396

Cantalapiedra CP, Hernández-Plaza A, Letunic I, Bork P, Huerta-Cepas J (2021) eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. Mol Biol Evol 38:5825–5829. https://doi.org/10.1093/molbev/msab293

Cazorla FM, Duckett SB, Bergström ET et al (2006) Biocontrol of avocado dematophora root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-Hexyl 5-Propyl Resorcinol. Mol Plant Microbe Interact 19:418–428. https://doi.org/10.1094/MPMI-19-0418

Chepsergon J, Moleleki LN (2023) Rhizosphere bacterial interactions and impact on plant health. Curr Opin Microbiol 73:102297. https://doi.org/10.1016/j.mib.2023.102297

Choi K-H, Kumar A, Schweizer HP (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64:391–397. https://doi.org/10.1016/j.mimet.2005.06.001

Cole BJ, Feltcher ME, Waters RJ et al (2017) Genome-wide identification of bacterial plant colonization genes. PLoS Biol 15:e2002860. https://doi.org/10.1371/journal.pbio.2002860

De Bentzmann S, Aurouze M, Ball G, Filloux A (2006) FppA, a novel *Pseudomonas aeruginosa* prepilin peptidase involved in assembly of type IVb pili. J Bacteriol 188:4851–4860. https://doi.org/10.1128/JB.00345-06

Giltner CL, Nguyen Y, Burrows LL (2012) Type IV pilin proteins: versatile molecular modules. Microbiol Mol Biol Rev 76:740–772. https://doi.org/10.1128/MMBR.00035-12

González-Sánchez MÁ, Pérez-Jiménez RM, Pliego C, Ramos C, de Vicente A, Cazorla FM (2010) Biocontrol bacteria selected by a direct plant protection strategy against avocado white root rot show antagonism as a prevalent trait. J Appl Microbiol 109:65–78. https://doi.org/10.1111/j.1365-2672.2009.04628.x

Hacquard S, Spaepen S, Garrido-Oter R, Schulze-Lefert P (2017) Interplay between innate immunity and the plant microbiota. Annu Rev Phytopathol 55:565–589. https://doi.org/10.1146/annurev-phyto-080516-035623

Heredia-Ponce Z, Gutiérrez-Barranquero JA, Purtschert-Montenegro G, Eberl L, de Vicente A, Cazorla FM (2021) Role of extracellular matrix components in the formation of biofilms and their contribution to the biocontrol activity of *Pseudomonas chlororaphis* PCL1606. Environ Microbiol 23:2086–2101. https://doi.org/10.1111/1462-2920.15355

Kachlany SC, Planet PJ, Bhattacharjee MK, Kollia E, Desalle R, Fine DH, Figurski DH (2000) Nonspecific adherence by *Actinobacillus actinomycetemcomitans* requires genes widespread in bacteria and archaea. J Bacteriol 182:6169–6176. https://doi.org/10.1128/JB.182.21.6169-6176.2000

Kachlany SC, Planet PJ, DeSalle R, Fine DH, Figurski DH (2001) Genes for tight adherence of *Actinobacillus actinomycetemcomitans*: from plaque to plague to pond scum. Trends Microbiol 9:429–437. https://doi.org/10.1016/S0966-842X(01)02161-8

Knights HE, Jorrin B, Haskett TL, Poole PS (2021) Deciphering bacterial mechanisms of root colonization. Environ Microbiol Rep 13:428–444. https://doi.org/10.1111/1758-2229.12934

Koprivova A, Kopriva S (2022) Plant secondary metabolites altering root microbiome composition and function. Curr Opin Plant Biol 67:102227. https://doi.org/10.1016/j.pbi.2022.102227

Kwak M-J, Kong HG, Choi K et al (2018) Rhizosphere microbiome structure alters to enable wilt resistance in tomato. Nat Biotechnol 36:1100–1109. https://doi.org/10.1038/nbt.4232

Laloux G (2020) Shedding light on the cell biology of the predatory bacterium *Bdellovibrio bacteriovorus*. Front Microbiol 10:3136. https://doi.org/10.3389/fmicb.2019.03136

Liu F, Yang S, Xu F, Zhang Z, Lu Y, Zhang J, Wang G (2022) Characteristics of biological control and mechanisms of *Pseudomonas chlororaphis* zm-1 against peanut stem rot. BMC Microbiol 22:9. https://doi.org/10.1186/s12866-021-02420-x

Liu F, Hu M, Tan X et al (2023) *Pseudomonas chlororaphis* L5 and *Enterobacter asburiae* L95 biocontrol Dickeya soft rot diseases by quenching virulence factor modulating quorum sensing signal. Microb Biotech 16:2145–2160. https://doi.org/10.1111/1751-7915.14351

Liu Y, Xu Z, Chen L, et al (2024) Root colonization by beneficial rhizobacteria. FEMS Microbiol Rev 48:fuad066. https://doi.org/10.1093/femsre/fuad066

Loper JE, Hassan KA, Mavrodi DV et al (2012) Comparative genomics of plant-associated Pseudomonas spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genet 8:e1002784. https://doi.org/10.1371/journal.pgen.1002784

Lopes LD, Hao J, Schachtman DP (2021) Alkaline soil pH affects bulk soil, rhizosphere and root endosphere microbiomes of plants growing in a Sandhills ecosystem. FEMS Microbiol Ecol 97:fiab028. https://doi.org/10.1093/femsec/fiab028

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918

Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461–490. https://doi.org/10.1146/annurev.phyto.39.1.461

Matas IM, Castañeda-Ojeda MP, Aragón IM et al (2014) Translocation and functional analysis of *Pseudomonas savastanoi* pv. savastanoi NCPPB 3335 type III secretion system effectors reveals two novel effector families of the *Pseudomonas syringae* complex. Mol Plant Microbe Interact 27:424–436. https://doi.org/10.1094/MPMI-07-13-0206-R

Mendes R, Garbeva P, Raaijmakers JM (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS

Microbiol Rev 37:634–663. https://doi.org/10.1111/1574-6976.12028

Molina-Santiago C, Pearson JR, Navarro Y et al (2019) The extracellular matrix protects *Bacillus subtilis* colonies from *Pseudomonas* invasion and modulates plant co-colonization. Nat Commun 10:1919. https://doi.org/10.1038/s41467-019-09944-x

Molina-Santiago C, Vela-Corcía D, Petras D et al (2021) Chemical interplay and complementary adaptative strategies toggle bacterial antagonism and co-existence. Cell Rep 36:109449. https://doi.org/10.1016/j.celrep.2021.109449

Montes-Osuna N, Gómez-Lama Cabanás C, Valverde-Corredor A, Berendsen RL, Prieto P, Mercado-Blanco J (2021) Assessing the involvement of selected phenotypes of *Pseudomonas simiae* PICF7 in olive root colonization and biological control of *Verticillium dahliae*. Plants 10:412. https://doi.org/10.3390/plants10020412

Nikolaidis M, Mossialos D, Oliver SG, Amoutzias GD (2020) Comparative analysis of the core proteomes among the *Pseudomonas* major evolutionary groups reveals species-specific adaptations for *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis*. Diversity 12:289. https://doi.org/10.3390/d12080289

Nykyri J, Mattinen L, Niemi O et al (2013) Role and regulation of the Flp/Tad pilus in the virulence of *Pectobacterium atrosepticum* SCRI1043 and *Pectobacterium wasabiae* SCC3193. PLoS ONE 8:e73718. https://doi.org/10.1371/journal.pone.0073718

O'Connell Motherway M, Zomer A, Leahy SC et al (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc Natl Acad Sci USA 108:11217–11222. https://doi.org/10.1073/pnas.1105380108

Pliego C, De Weert S, Lamers G, de Vicente A, Bloemberg G, Cazorla FM, Ramos C (2008) Two similar enhanced root-colonizing Pseudomonas strains differ largely in their colonization strategies of avocado roots and *Rosellinia necatrix* hyphae. Environ Microbiol 10:3295–3304. https://doi.org/10.1111/j.1462-2920.2008.01721.x

Polonio Á, Vida C, de Vicente A, Cazorla FM (2017) Impact of motility and chemotaxis features of the rhizobacterium *Pseudomonas chlororaphis* PCL1606 on its biocontrol of avocado white root rot. Int Microbiol 20:95–104. https://doi.org/10.2436/20.1501.01.289

Pu M, Rowe-Magnus DA (2018) A Tad pilus promotes the establishment and resistance of Vibrio vulnificus biofilms to mechanical clearance. NPJ Biofilms Microbiomes 4:10. https://doi.org/10.1038/s41522-018-0052-7

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341–361. https://doi.org/10.1007/s11104-008-9568-6

Santoyo G, Urtis-Flores CA, Loeza-Lara PD, Orozco-Mosqueda MDC, Glick BR (2021) Rhizosphere colonization determinants by plant growth-promoting rhizobacteria (PGPR). Biology 10:475. https://doi.org/10.3390/biology10060475

Sarraf M, Janeeshma E, Arif N et al (2023) Understanding the role of beneficial elements in developing plant stress resilience: signalling and crosstalk with phytohormones and microbes. Plant Stress 10:100224. https://doi.org/10.1016/j.stress.2023.100224

Schilling J, Wagner K, Seekircher S, Greune L, Humberg V, Schmidt MA, Heussip G (2010) Transcriptional activation of the tad type IVb pilus operon by PypB in *Yersinia enterocolitica*. J Bacteriol 192:3809–3821. https://doi.org/10.1128/JB.01672-09

Schreiner HC, Sinatra K, Kaplan JB et al (2003) Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. Proc Natl Acad Sci USA 100:7295–7300. https://doi.org/10.1073/pnas.1237223100

Seef S, Herrou J, De Boissier P et al (2021) A Tad-like apparatus is required for contact-dependent prey killing in predatory social bacteria. eLife 10:e72409. https://doi.org/10.7554/eLife.72409

Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW (2011) Pseudomonas genomes: diverse and adaptable. FEMS Microbiol Rev 35:652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x

Solomon W, Janda T, Molnár Z (2024) Unveiling the significance of rhizosphere: implications for plant growth, stress response, and sustainable agriculture. Plant Physiol Biochem 206:108290. https://doi.org/10.1016/j.plaphy.2023.108290

Sorroche FG, Spesia MB, Zorreguieta A, Giordano W (2012) A positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from Argentina. Appl Environ Microbiol 78:4092–4101. https://doi.org/10.1128/AEM.07826-11

Stover CK, Pham XQ, Erwin AL et al (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406:959–964. https://doi.org/10.1038/35023079

Thiery S, Kaimer C (2020) The predation strategy of *Myxococcus xanthus*. Front Microbiol 11:2. https://doi.org/10.3389/fmicb.2020.00002

Thiery S, Turowski P, Berleman JE, Kaimer C (2022) The predatory soil bacterium *Myxococcus xanthus* combines a Tad- and an atypical type 3-like protein secretion system to kill bacterial cells. Cell Rep 40:111340. https://doi.org/10.1016/j.celrep.2022.111340

Tienda S, Gutiérrez-Barranquero JA, Padilla-Roji I, Arrebola E, de Vicente A, Cazorla FM (2024) Polyhydroxyalkanoate production by the plant beneficial rhizobacterium *Pseudomonas chlororaphis* PCL1606 influences survival and rhizospheric performance. Microbiol Res 278:127527. https://doi.org/10.1016/j.micres.2023.127527

Tomich M, Planet PJ, Figurski DH (2007) The tad locus: postcards from the widespread colonization island. Nat Rev Microbiol 5:363–375. https://doi.org/10.1038/nrmicro1636

Tran H, Ficke A, Asiimwe T, Höfte M, Raaijmakers JM (2007) Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. New Phytol 175:731–742. https://doi.org/10.1111/j.1469-8137.2007.02138.x

Trunk TS, Khalil HS, Leo JC (2018) Bacterial Autoaggregation. AIMS Microbiol 4:140–164. https://doi.org/10.3934/microbiol.2018.1.140

Vo JL, Ortiz GCM, Totsika M et al (2022) Variation of Antigen 43 self-association modulates bacterial compacting within aggregates and biofilms. NPJ Biofilms Microbiomes 8:20. https://doi.org/10.1038/s41522-022-00284-1

Wang D, Dorosky RJ, Han CS et al (2015) Adaptation genomics of a small-colony variant in a *Pseudomonas chlororaphis* 30–84 Biofilm. Appl Environ Microbiol 81:890–899. https://doi.org/10.1128/AEM.02617-14

Wang H, Zhang Y, Dai D et al (2024) Genomic insight into the insecticidal potential of a new *Pseudomonas chlororaphis* isolate. J Econ Entomol 117:82–92. https://doi.org/10.1093/jee/toad232

Wei D, Zhu D, Zhang Y et al (2024) *Pseudomonas chlororaphis* IRHB3 assemblies beneficial microbes and activates JA-mediated resistance to promote nutrient utilization and inhibit pathogen attack. Front Microbiol 15:1328863. https://doi.org/10.3389/fmicb.2024.1328863

Wheatley RM, Poole PS (2018) Mechanisms of bacterial attachment to roots. FEMS Microbiol Rev 42:448–461. https://doi.org/10.1093/femsre/fuy014

Yin C, Casa Vargas JM, Schlatter DC, Hagerty CH, Hulbert SH, Paulitz TC (2021) Rhizosphere community selection reveals bacteria associated with reduced root disease. Microbiome 9:86. https://doi.org/10.1186/s40168-020-00997-5

Yuan J, Zhao J, Wen T et al (2018) Root exudates drive the soil-borne legacy of aboveground pathogen infection. Microbiome 6:156. https://doi.org/10.1186/s40168-018-0537-x

Zboralski A, Filion M (2020) Genetic factors involved in rhizosphere colonization by phytobeneficial *Pseudomonas spp*. Comput Struct Biotechnol J 18:3539–3554. https://doi.org/10.1016/j.csbj.2020.11.025

Zeriouh H, De Vicente A, Pérez-García A, Romero D (2014) Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. Environ Microbiol 16:2196–2211. https://doi.org/10.1111/1462-2920.12271

Zumaquero A, Macho AP, Rufián JS, Beuzón CR (2010) Analysis of the role of the type III effector inventory of *Pseudomonas syringae pv. phaseolicola* 1448a in interaction with the plant. J Bacteriol 192:4474–4488. https://doi.org/10.1128/JB.00260-10