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Impact of motility and chemotaxis features of the rhizobacterium *Pseudomonas chlororaphis* PCL1606 on its biocontrol of avocado white root rot

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Summary. The biocontrol rhizobacterium *Pseudomonas chlororaphis* PCL1606 has the ability to protect avocado plants against white root rot produced by the phytopathogenic fungus *Rosellinia necatrix*. Moreover, PCL1606 displayed direct interactions with avocado roots and the pathogenic fungus. Thus, nonmotile (*flgK* mutant) and non-chemotactic (*cheA* mutant) derivatives of PCL1606 were constructed to emphasize the importance of motility and chemotaxis in the biological behaviour of PCL1606 during the biocontrol interaction. Plate chemotaxis assay showed that PCL1606 was attracted to the single compounds tested, such as glucose, glutamate, succinate, aspartate and malate, but no chemotaxis was observed to avocado or *R. necatrix* exudates. Using the more sensitive capillary assay, it was reported that smaller concentrations (1 mM) of single compounds elicited high chemotactic responses, and strong attraction was confirmed to avocado and *R. necatrix* exudates. Finally, biocontrol experiments revealed that the *cheA* and *fglK* derivative mutants reduced root protection against *R. necatrix*, suggesting an important role for these biological traits in biocontrol by *P. chlororaphis* PCL1606. [**Int Microbiol** 20(2):94-104 (2017)]

Keywords: *Pseudomonas chlororaphis* · *Rosellinia necatrix* · avocado white root rot · multitrophic interactions · rhizosphere

Introduction

Plant roots serve a multitude of functions in the plant, including anchorage, the provision of nutrients and water, and the production of exudates with growth regulatory properties. All plant roots have the remarkable ability to secrete both low- and high-molecular-weight molecules into the rhizosphere [2]. Root exudation includes the release of ions, as well as oxygen and water, but root exudates mainly consist of a mixture of carbon-containing compounds derived from the products of photosynthesis, thus influencing plant growth and soil ecology [4]. One of the main impacts of root exudates is on the soil microbial community in their immediate vicinity, influencing pest resistance, supporting beneficial symbiosis, altering the chemical and physical properties of the soil and inhibiting the growth of competing plant species [4].

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The composition of root exudates varies with plant species, environmental conditions and even with agricultural managements, causing a nutrient gradient in the soil with attractive activity to motile bacteria [39,45], and stimulating the selection of different members of the rhizospheric microbial community [3,47]. It is well known that plant root exudates can be considered the primary sources of carbon and energy in the rhizosphere [35,36,42,47]. Most motile bacteria can sense and respond to low concentrations of organic compounds in this environment by the process of chemotaxis [20], defined as the ability of motile bacteria to direct their movement in gradients of chemorepellents and chemoattractants [39]. Thus, chemotaxis has been suggested to be the first step in the bacterial colonization of roots of several plant species [44]. Furthermore, an enrichment in the motile bacteria with chemotaxis-encoding genes in the rhizosphere compared to the bulk soil, have been reported in previous studies [39]. In this case, bacterial chemotaxis provides a competitive advantage to motile flagellated bacteria in root colonization as an essential characteristic for the improvement of plant health [2]. Both motility and chemotaxis could then be considered key characteristics of plant-growth-promoting rhizobacteria and could thus play important roles in the interactive process that occurs in the soil [1].

One of the beneficial interactions among plant and bacterial, is the promotion of plant growth, where the chemotaxis of plant-growth-promoting rhizobacteria (PGPR) towards the roots system occurs prior to the colonization [12]. The microbial colonization is one of the most important aspects in the plant-bacteria interactions, since the success in the colonization is considered as an initial step in the protection of plants from soilborne pathogens [15,42]. For example, many *Bacillus* [48] and *Pseudomonas* [25,28,46] strains which have been used as biocontrol agents and/or to induce plant growth, have been described as flagellar motile bacteria to root exudates.
In general, pseudomonads can sense chemical gradients and respond to them using flagella or pili coupled to a chemosensory system with multiple copies of chemosensory genes [38]. For example, de Weert et al. [14], described that *Pseudomonas fluorescens* WCS365 is an excellent competitive colonizer of tomato root tips with biocontrol activity against tomato foot and root rot (TFRR), caused by the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. In this case, the strain WCS365 was able to colonize both, the tomato roots [15] and the fungal hyphae, in order to control the disease [14].

The biocontrol rhizobacterium *Pseudomonas chlororaphis* PCL1606 has protective features against different soilborne phytopathogenic fungi, including *Rosellinia necatrix*, which is the causal agent of avocado white root rot, and *Fusarium oxysporum* f. sp. *radicis-lycopersici*, which is the causal agent of tomato crown and foot rot [10,13]. The production of the antifungal antibiotic 2-hexyl-5-propyl-resorcinol (HPR) is considered the major factor responsible for the antagonistic phenotype of this rhizobacterium [10]. Moreover, additional studies have demonstrated a crucial role for HPR in the biocontrol of *R. necatrix* and *F. oxysporum* [9,19]. This bacterium has demonstrated efficient colonization of avocado roots, where it can be established at least for several weeks. Interestingly, during the multitrophic interactions that occur during biocontrol events, PCL1606 can also directly interact with the pathogenic fungus *R. necatrix*, suggesting a possible role for exudates from plant roots and/or fungal mycelium in such efficient interactions [7].

The aim of the present study was to analyse the potential involvement of motility and chemotaxis in the biocontrol activity to better understand the biology of the multitrophic interactions of *P. chlororaphis* PCL1606.

Materials and methods

Microorganisms and growth conditions. The microorganisms used in the present study are listed in Table 1. The wild-type rhizobacterium *Pseudomonas chlororaphis* PCL1606 was isolated from avocado roots [10]. Its genome sequence is available from the NCBI database (NCBI accession number CP011110.1; [9]). Luria-Bertani medium (LB) was routinely used to culture *Pseudomonas* strains at 25 °C. Agar (Difco Laboratories, Detroit, MI, USA) was added to a final concentration of 1.5% to produce solid medium. The medium was supplemented with kanamycin (50 µg/ml) when using or selecting derivatives containing the plasmid pCR2.1 (InvitroGen, Waltham, MA, USA). The bacterial strains were stored at –80 °C in LB with 10% dimethyl sulfoxide.

In this study, the virulent strain *Rosellinia necatrix* CH53 was used [33]. The fungus was grown at 25 °C on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). For preservation, microsclerotia of the fungal strain were stored in TPG at 4 °C as previously described [22].

Construction of *Pseudomonas chlororaphis* PCL1606 insertional mutants. The genome sequence of *P. chlororaphis* PCL1606, allowed the identification of only one functional *cheA* gene, and a derivative mutant on it was constructed in order to obtain an impaired strain in chemotactic abilities. Additionally, and to be used as a negative control in experimentation, a derivative mutant in motility was also obtained in *flgK* gene. PCR, cloning and plasmid purification were performed following standard procedures [37]. The selected *cheA* and *flgK* genes of *P. chlororaphis* PCL1606 (loci AKA25886.1 and AKA25937, respectively) were inactivated by insertional mutagenesis as previously described [8]. To accomplish this

Table 1. Main characteristics of bacterial and fungal strains, and plasmids used in this study

Strain / Plasmid	Relevant characteristics[a]	Reference
Bacteria		
Pseudomonas chlororaphis		
PCL1606	Wild-type, isolated from Spanish avocado rhizosphere, motility +, antagonism +	[10]
PCL1606-*cheA*	PCL1606 derivative insertional mutant in *cheA* gene, motility +, antagonism +, Km^r	This study
PCL1606-*flgK*	PCL1606 derivative insertional mutant in *flgK* gene, motility -, antagonism +, Km^r	This study
Pseudomonas fluorescens		
Pf0-1	Wild-type, Motility +, antagonism +	[13]
Escherichia coli		
DH5α	General-purpose *Escherichia coli* host strain	[6]
Fungi		
Rosellinia necatrix		
CH53	Wild-type, isolated from avocado trees with symptoms of white root rot; high virulence	[33]
Plasmids		
pCR2.1-TOPO	Cloning vector for PCR products; used to construct mutants in *cheA* and *flgK*. Amp^r, Km^r	TA Cloning Kit, Invitrogen, UK

[a]Motility: spreading behaviour of a bacterial suspension (10 µl of 1.5×10^7 cfu/ml) on diluted KB soft agar [23]. + = motility, and – = no motility. Antagonism: bacterial strains showing a mycelial inhibition zone of *Rosellinia necatrix* after 5 days of growth on TPG agar plates [10]. + = antagonistic, and – = non antagonistic. Antibiotic-resistance: Km^r = kanamycin, Amp^r = ampicillin.

inactivation, vectors were constructed via insertion to disrupt the *cheA* and *flgK* genes using single-crossover homologous recombination. To construct the integrative plasmids, DNA fragments of 565 and 477 bp from inside the open reading frame of the *cheA* and *flgK* genes, respectively, were obtained using specific PCR primers (Table 2), and DNA from *P. chlororaphis* PCL1606 was used as a template. The amplified DNA fragments were then cloned independently into the pCR2.1-TOPO vector on *E. coli* DH5α (Table 1). Subsequently, these integrative plasmids were isolated and transformed into electrocompetent wild-type *P. chlororaphis* PCL1606 cells using standard electroporation [11]. Five colonies from each independent transformation assay were randomly selected, and the correct insertion and orientation of the plasmid within the target gene were confirmed by PCR using the primers described in Table 2, followed by sequencing of the amplified DNA to confirm gene disruption. The resulting derivative mutants were named PCL1606-*cheA* and PCL1606-*flgK* (Table 1). The insertional mutants were selected in the presence of kanamycin (50 µg/ml). Growth characterization on M9 minimal medium [34] and LB, compared with the wild-type strain, was performed to confirm that these derivative mutants did not have altered growth.

Antagonistic activity. The antagonistic activity of rhizobacterial wild-type PCL1606 and derivative isolates (PCL1606-*cheA* and PCL1606-*flgK*) was tested as described previously [10]. Initial screening for in vitro antifungal activity on LB and PDA agar plates against *R. necatrix* CH53 was performed by placing on the agar in the centre of a Petri dish a 0.6-cm diameter mycelium disk from a 5-day-old fungal culture grown at 25 °C, followed by inoculation of the bacterial strains at a distance of approximately 3 cm from the fungus. Bacterial strains inhibiting mycelial growth after five days of growth, as judged by a growth inhibition zone, were considered antagonistic.

Motility and chemotaxis plate assays. Motility assay was performed essentially as described previously [16], using plates of King's B medium [23] twenty times diluted and amended with 0.3% agar. A bacterial suspension (10 µl drop containing approximately 1.5×10^7 cfu/ml) was placed in the center of the plate and was incubated for 24 h at 25 ºC. Halo diameters of motility were recorded, and additionally, the motility of the bacteria into the suspension was also confirmed under light microscopy (100×).

Plate chemotaxis assay was performed as previously described [35] with slight modifications and using M9 minimal medium containing 0.2% Noble agar (Difco) and no carbon source. Ten microliters of a bacterial suspension (approximately 10^7 cfu/ml), obtained from an overnight culture on M9 plates with glucose or glycerol (10 mM), was placed in the center of a chemotaxis plate. Then, three separate drops (10 µl each) of the compound to be tested as the chemoattractant carbon source were placed on the right side of the interior of the plate, and on the left side, as a control for no attraction, three separate drops (10 µl each) of sterile saline solution were placed. The plates were then incubated at 25 ºC for 36 h. The experiments were performed in triplicate.

Single compounds assayed by the chemotaxis plate assay included L-glutamate, and mix of D- and L-glucose, succinate, aspartate and malate (Sigma, St Louis, MO, USA) as representative compounds previously reported to be present in the plant rhizosphere [2,4,15,26,35,40]. Different concentrations of the test compounds were assayed (from 1 mM to 100 mM).

Avocado root exudates and *R. necatrix* exudates were also tested. Exudates from avocado were obtained as follows. One-month-old commercial avocado seedlings (var. Walter Hole) were provided by Brokaw nursery (Brokaw España, Vélez Málaga, Spain). The plant was removed from the pots, and the roots were washed and disinfected as previously described [10]. Avocado cotyledons were carefully removed, and these plants were then in-

Table 2. Primer sequences and use in this study

Primer	Sequence	Use
MutcheA-F	5′-CACATCCTGTTGTCGATCTC	Used to partially amplify the genes *cheA* and *flgK* of PCL1606, in order to be used for construction of insertional mutants
MutcheA-R	5′-GTTGATCGACGACGAAGCCG	
MutflgK-F	5′-AACATCAACGGCAACCTGAG	
MutflgK-R	5′-GTTCTTGTCGATACCCTGGC	
M13-F	5′-GTAAAACGACGGCCAGT	Used to determine orientation of the insert in the constructed derivative mutants by insertion. M13 primers from vector pCR2.1, and external primers on the sequence or each gen
M13-R	5′-CAGGAAACAGCTATGAC	
ExMutcheA-F	5′-AGGAAGCTTAGATCTGGTTGCGGCTGTAG	
ExMutcheA-R	5′-TGCTCTAGACATCCATCCATATCGGCATCTCG	
ExMutflgk-F	5′-GCCAAGCTTACAACCTGTTCGGCATCAAG	
ExMutflgK-R	5′-GAATCTAGAGTTCTTCTGATAGTTGGCGG	

troduced into gnotobiotic tubes, as similarly described in a previous work [41]. Gnotobiotic avocados were placed into a growth chamber (25 ºC, 16:8 h of light:dark) and were watered every other day with 8–10 ml of sterile distilled water. After 10 days, the clear exudates were recovered from the bottoms of the tubes, were sterilized by filtration (0.2 µm pore diameter, ©Merck Millipore, Darmstadt, Germany), and were preserved in the dark at –20 ºC. To obtain *R. necatrix* exudates, the method previously described was followed [32]. Briefly, the fungus *R. necatrix* CH53 was grown at 25 ºC on BM [25] agar plates until the plates were completely covered by the fungus. Pieces of fungal mycelia were collected from one plate and were inoculated on 100 ml of minimal medium BM. The culture was incubated at 25 ºC for 2 weeks without shaking. Exudates obtained from the culture medium were obtained by filtration using ALBET filter paper in reams of 73 g/m and were preserved in the dark at –20 ºC. To avoid low concentration of both exudates, fractions of 20 ml of avocado and *R. necatrix* exudates were concentrated 20 times with a speed vacuum (Savant SpeedVac SCV100M, Thermo Fisher Scientific, USA) for further experimentation.

Modified capillary chemotaxis assay. The chemotaxis capillary assay with some modifications from the previous description [29] was performed using a disposable 200 µl pipette tip as a chamber to hold 100 µl of bacterial suspension (usually 1×10^7 cells) in sterile saline solution (0.85% NaCl). A 13 mm needle with inner diameter of 0.3 mm (Becton Dickinson, Ireland) was used as the chemotaxis capillary and was attached to a 1 ml tuberculin syringe (Becton Dickinson) containing 100 µl portion of the compound (1 mM to 100 mM) to be tested in sterile saline solution. As controls, saline solution and a solution of casamino acids (10%) were used. Casamino acids are an attractant of *Pseudomonas* sp. Pf0-1, which was also used as a control bacterial strain for chemotaxis [35]. After 45 min of incubation at 25 ºC, the needle syringe was removed from the bacterial suspension, and the contents were diluted and plated in LB medium. Accumulation of bacteria in the capillaries was calculated as the average of the bacterial counts obtained in triplicate from the plates, and the results were expressed as the means of at least three separate capillary assays for each determination. The relative chemotaxis index (RCI) was calculated as the ratio of the bacteria that entered the test capillary to that in the control capillary with saline solution. An RCI of 2 or greater has been described as significant with this method [29].

Biocontrol. The roles of motility and chemotaxis in biocontrol were evaluated. Biocontrol assays against avocado white root rot were performed using the avocado-*R. necatrix* system, as previously described [10]. Six-month-old commercial avocado plants (cv. Walter Hole) were obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain). The roots of the avocado plants were disinfected by immersion in 0.1% NaOCl for 20 min and then were washed and bacterized following the method previously described [10], with slight modifications. The roots of the avocado plants were immersed in a suspension of the bacterial isolate (10^9 cfu/ml) or in sterile tap water for 20 min. For these experiments, a rifampicin resistant-derivative of PCL1606 was used [13,19]. Any excess bacterial suspension was allowed to drip off, after which the seedlings were placed into pots containing 30 g of wet potting soil (Jongkind Grond B.V., Aalsmeer, the Netherlands) and were infected with *R. necatrix* CH53 using inoculated wheat grains (four infected grains per pot), as described previously [18]. Three sets of five avocado seedlings each were tested per treatment. The seedlings were grown in a chamber at 24 °C and 70% relative humidity with 16 h of daylight, and they were watered twice per week. Because it was difficult to monitor the symptoms on the avocado roots due to the overgrowth of *R. necatrix*, aerial symptoms were recorded on a scale of 0 to 3, and a disease index (DI) was calculated using a previously described formula [10]. The DI was determined approximately 21 days after bacterization.

To report whether the mutations in motility and chemotaxis had effects on bacterial survival on the roots, bacterial counts were performed at the end of the biocontrol assays. Avocado root samples were gently shaken to remove loosely adhering soil and were aseptically transferred to sterile bags and immediately processed. Roots from each set of five avocado seedlings were combined in the same bag and were analysed (3 samples per treatment). Root samples were washed twice in tap water, weighed and homogenized in a stomacher (Colworth Stomacher-400, Seward Ltd.) for 3 min with 10 ml of sterile phosphate-buffered saline (PBS, pH 7.2) per gram of fresh root material. Suspensions were diluted 10-fold and were plated on TPG amended with cycloheximide (100 µg/ml) to prevent fungal growth or amended or not with kanamycin (50 µg/ml) or rifampicin (50 µg/ml). The plates were incubated at 24 °C for 48 h. After incubation, the numbers of antibiotic-resistant bacterial colonies with the typical morphology were recorded.

Results

Motility assay in swim plates. To study the importance of motility in accessing some of the compounds that *P. chlororaphis* PCL1606 could transform or use as carbon sources, we constructed mutants impaired in motility (PCL1606-*flgK*) and in chemotaxis (PCL1606-*cheA*) for *P. chlororaphis* PCL1606. Characterization of the constructed derivative mutants revealed that no effect on growth rate in TPG was observed. Checking the motility of these microorganisms in soft agar plates of KB medium diluted twenty times, a clear motility response was observed for PCL1606 (Fig. 1). The swimming behaviour of *P. chlororaphis* PCL1606 in plate assay was very similar to that displayed by the also motile *Pseudomonas* sp. Pf0-1 strain. The motility of PCL1606-*cheA* was statistically less than the wild-type strain PCL1606 but still motile, as also confirmed by microscopic analysis. In contrast, the PCL1606-*flgK* mutant was not motile (Fig. 1), neither by plate assay nor by microscopic analysis.

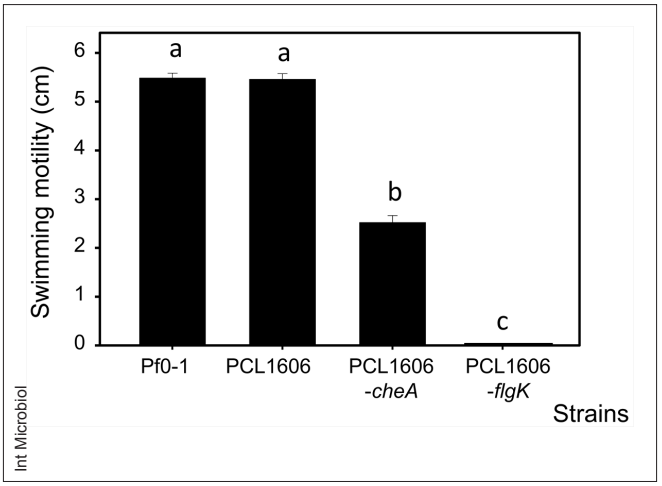

Fig. 1. Swimming motility of the wild-type strains *Pseudomonas* sp. Pf0-1 and *Pseudomonas chlororaphis* PCL1606, and mutant derivatives mutants PCL1606-*cheA* and PCL1606-*flgK*, constructed by gene insertion. Ten µl of a bacterial suspension (1.5×10^7 cells/ml) were placed in the centre of the plate containing King's B medium diluted twenty times. Movement was recorded after incubation at 25 ºC during 24 h, and disk area of motility was calculated. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant test ($P = 0.05$). Values of bars with different letter indications denote a statistically significant difference.

Chemotactic responses of *P. chlororaphis* PCL1606 to different compounds. Using the chemotaxis plate assay, *P. chlororaphis* PCL1606 displayed a clear attraction and motility towards casamino acids, as well as the positive control strain *Pseudomonas* sp. Pf0-1. No chemotactic movement was observed in any case for the PCL1606-*cheA* and PCL1606-*flgK* derivative mutants (Fig. 2A). Individual compounds (L-glutamate and mixtures of D- and L-aspartate, succinate, malate and glucose) were tested as attractants at different concentrations (1, 10, 40 and 100 mM; Fig. 2B). Attraction at different levels to all of the compounds was observed for Pf0-1 and PCL1606 compared with the negative control of attraction (sterile saline solution). A repulsion phenotype was observed at high concentrations of some compounds, especially for aspartate and L-glutamate at 100 mM (Fig. 2B). Bacterial chemotaxis increased with an increase in compound concentration (with a maximum of approximately 10–40 mM of concentration). Concentrations of 100 mM did not increase attraction, showing that, at such concentrations, these compounds could act as repellents for PCL1606. However, no chemotactic activity to root and fungal compounds was observed by the chemotaxis plate assay.

These results were complemented using the more accurate capillary chemotaxis assay for those individual compounds commonly present on plant roots. The capillary assays showed that *P. chlororaphis* PCL1606 cells accumulated preferably in capillaries containing lower concentrations with the maximum number of cells obtained at 1 mM. Concentrations greater than 10 mM did not show attraction or show themselves to be repellent for the tested compounds, except for glucose, which was still an attractant at 100 mM (data not shown). Testing these compounds at 1 mM of concentration showed that glucose and L-glutamate concentrated a greater number of cells inside the capillary, with RCI values of 8.1 and 7.5, respectively (Fig. 3). No chemotactic response was found for PCL1606-*cheA* and PCL1606-*flgK* derivative mutants impaired in chemotaxis and motility, respectively.

When using *R. necatrix* and avocado root exudates, cells were clearly attracted and concentrated inside the capillary needle. A strong attraction was observed to the avocado exudates (with an RCI of 12.5) but also to the *R. necatrix* exudates (RCI of 3.75). The use of more concentrated exudates (20×) resulted in a stronger chemotactic phenotype (Fig. 4). No chemotaxis derived from the media used to obtain the *R. necatrix* exudates (BM medium) was observed (Fig. 5).

Biocontrol experiments. The biocontrol experiments, performed with 6-month-old commercial avocado plants under greenhouse conditions, revealed high biocontrol activity of the wild-type strain *P. chlororaphis* PCL1606 (Fig. 6). However, the disease index of the derivative mutants, im-

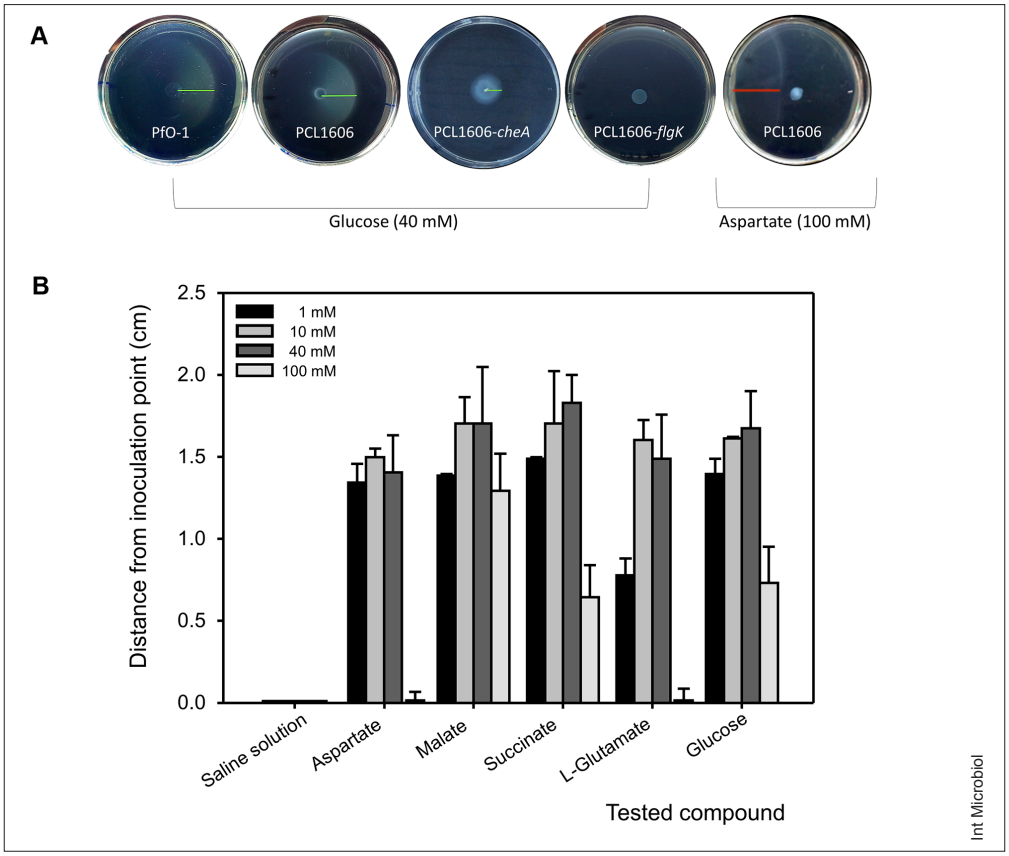

Fig. 2. Chemotaxis assay on plate. a) Visualization of chemotactic response on plate assay towards glucose (40 mM) of different *Pseudomonas* sp. Ten µl of a bacterial suspension was allocated at the centre of the plate, and three 10 µl separate drops of the test solution at the right side of the plate. The strain of *Pseudomonas* sp. Pf0-1 was used as positive control of chemotaxis. b) Chemotactic response by plate assay of *P. chlororaphis* PCL1606 towards different individual compounds and concentrations. Error bars indicated the SDs based in three independent experiments.

paired in motility (PCL1606-*flgK*) and chemotaxis (PCL1606-*cheA*), was significantly affected in biocontrol compared with the wild-type strain PCL1606. However, those derivative mutants still displayed antagonism and biocontrol ability (Table 1) but with significantly lower levels of protection (Fig. 6).

Bacterial counts on avocado roots after 21 days of growth were analysed. The bacterial counts of the rifampicin-resistant wild-type strain PCL1606 (1.5×10^6 cfu/g of fresh root) and its derivative mutants (1.1×10^6 cfu/g of fresh root for PCL1606-*cheA*, and 2.3×10^6 cfu/g of fresh root for PCL1606-*flgK*) revealed non-significant differences ($P = 0.05$) among them after ANOVA.

Discussion

Plant biocontrol activity is the result of multitrophic interactions among, at least, a susceptible host plant, a phytopathogenic agent and a beneficial organism. Plant roots are able to exude an extensive range of organic compounds, which can function as nutrients or carbon sources for microorganisms. This ability is one of the reasons why the rhizosphere is inhabited by a broad range of microorganisms. Unfortunately, not only beneficial microbes are attracted by root exudate but also microorganisms that can damage the plant [2].

Pseudomonas chlororaphis PCL1606 is a beneficial bacterium that can be stablished on avocado root for several weeks, and it is able to interact directly with the root and with phytopathogenic fungi [7]. Motility analysis revealed that *P. chlororaphis* showed swimming motility, which is considered a common trait of several biocontrol *Pseudomonas* sp. [15,46]. Many pseudomonads, like *P. aeruginosa* [21], *P. syringae* and strains of *P. fluorescens* [30], have more than one functional chemotaxis system. However, in the case of *P. chlororaphis* PCL1606, only one functional chemotactic *cheA* gene has been identified (locus AKA25886.1). The construction of the non-chemotactic *cheA* and the flagella-less *flgK* mutants of

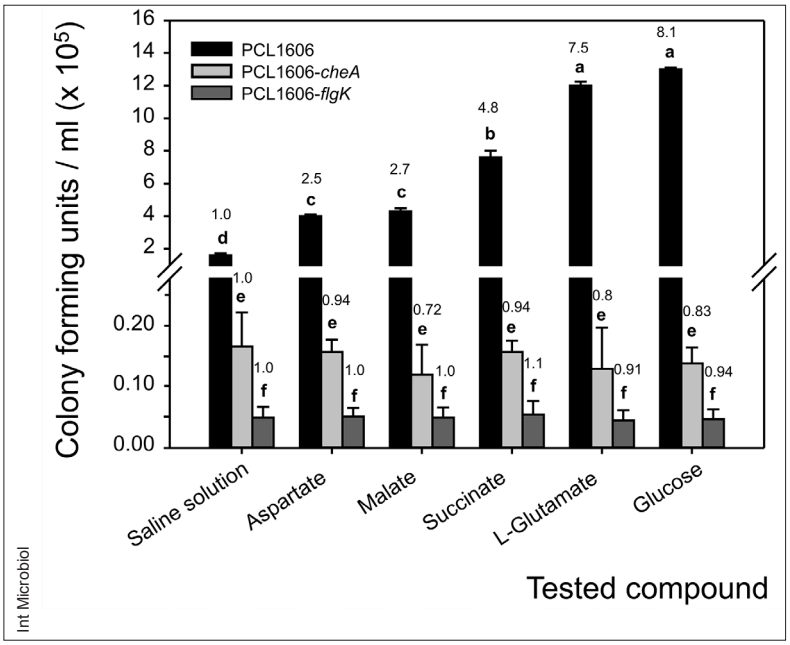

Fig. 3. Chemotaxis response of *Pseudomonas chlororaphis* PCL1606 to single compounds (1 mM) by capillary assay. A bacterial suspension was allocated into the capillary and immersed into a bacterial suspension for 45 min. Afterwards, bacterial counts were calculated. Error bars indicated the SDs based in three independent experiments. Numbers above each bar indicates the relative chemotactic index (RCI). RCI values equal or above 2 indicate positive chemotactic response. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant test ($P = 0.05$). Values of bars with different letter indications denote a statistically significant difference.

P. chlororaphis PCL1606 resulted in lack of response of these two derivative strains to chemotactic assay. Potential effects of the insertion on downstream genes cannot discarded; however, the genes susceptible to be affected in PCL1606-*cheA* and PCL1606-*flgK* would be the genes *cheY* and *flgL*, respectively, also involved in the same phenotype. It is important to indicate that the *cheA* mutant retained motility, in agreement with results previously described for other PCL1606-*cheA* mutants of biocontrol *Pseudomonas* sp. [15].

By chemotaxis plate assay, the rhizobacterium *P. chlororaphis* PCL1606 was attracted to all of these individual compounds. The tested individual compounds selected have been described to be found in plant root exudates, and they include sugars and simple polysaccharides, such as glucose, amino acids, such as aspartate and L-glutamate and organic acids, such as malate or succinate [4,26,40,41]. Amino acids and organic acid fractions of root exudates have been described as very important for *Pseudomonas* sp. chemotaxis [31,42], and some of them have been considered among the major chemoattractants for *Pseudomonas* sp. cells in the tomato rhizosphere [15]. The observed increase in the chemotaxis response when increasing the attractant concentration in some cases (1 mM, 10 mM, and in some cases 40 mM) suggested that this process might enable bacteria to detect a concentration gradient of at-

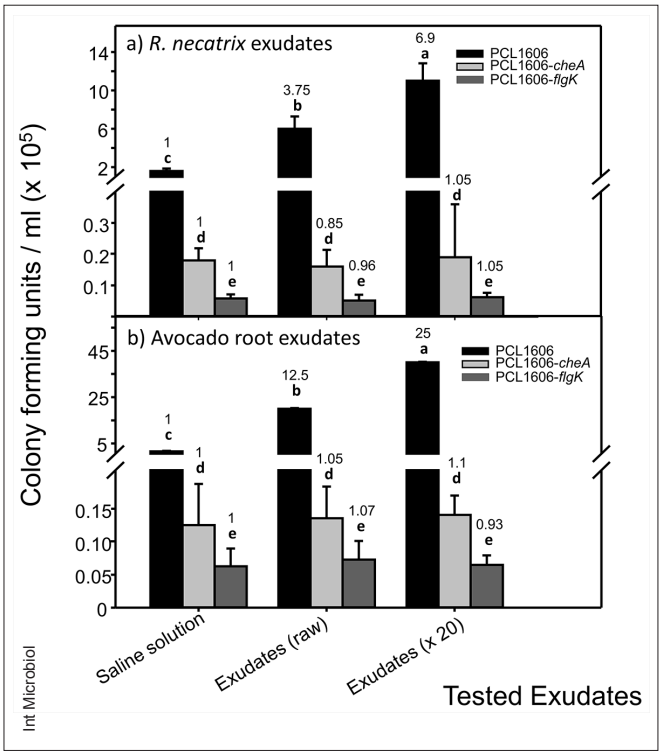

Fig. 4. Chemotaxis response by capillar assay of *Pseudomonas chlororaphis* PCL1606 and its derivatives to (**A**) *Rosellinia necatrix* and (**B**) avocado exudates. Error bars indicated the SDs based in three independent experiments. Numbers above each bar indicate the relative chemotactic index (RCI). RCI values equal or above 2 indicate positive chemotactic response. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant test ($P = 0.05$). Values of bars with different letter indications denote a statistically significant difference.

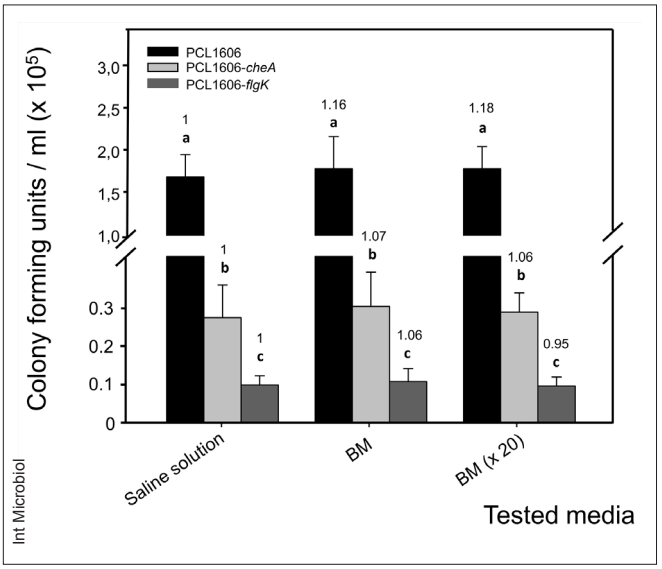

Fig. 5. Chemotaxis response by capillary assay of *Pseudomonas chlororaphis* PCL1606 to BM minimal medium used to obtain *Rosellinia necatrix* CH53 exudates. Error bars indicated the SDs based in three independent experiments. Numbers above each bar indicates the relative chemotactic response (RCI). RCI values equal or above 2 indicate positive chemotactic response. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant test ($P = 0.05$). Values of bars with different letter indications denote a statistically significant difference.

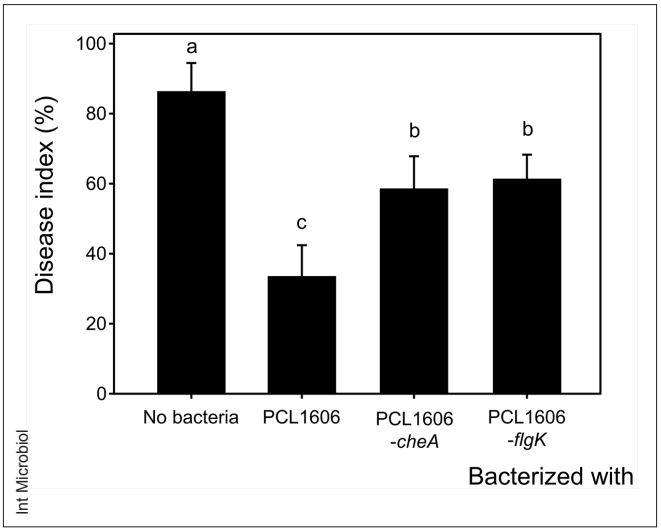

Fig. 6. Biocontrol of avocado white root rot caused by *Rosellinia necatrix* by *Pseudomonas chlororaphis* PCL1606 and derivatives impaired in chemotaxis (PCL1606-*cheA*) and in flagellar motility (PCL1606-*flgK*). Roots of avocado seedlings were inoculated with the different strains before transferring them to potting soil infested with *R. necatrix*. Plants were scored as sick or healthy after 21 days of growth after bacterization. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test ($P = 0.06$). Values of bars with different letter indications denote a statistically significant difference.

tractant and to reach the immediate vicinity of the roots. However, as previously described, the development of similar-size chemotactic rings in different concentrations (e.g., aspartate at 1, 10 and 40 mM) is difficult to explain [29]. Higher concentrations tested (100 mM) act as repellents for PCL1606, likely due to local changes in pH, as previously reported [24]. The lack of chemotaxis response of PCL1606 by plate assay when using complex root and fungal exudates as attractants could be due to some interference among the components present in the exudates and the agar media. The presence of a white precipitate could be the result of a reduction in solubility and precipitation of some components, and absence of available compounds.

However, because the plate assay is not considered ideal for quantifying bacterial migration [17], the use of the capillary assay showed greater sensitivity at lower concentrations (1 mM), thus revealing cell responses to compound concentrations closer to those present in nature [4,26]. The calculation of the RCI at a concentration of 1 mM has been extensively studied [20,29]. Nevertheless, slightly higher concentrations, such as 5 mM, have also been reported [30]. By capillary chemotaxis assay of *P. chlororaphis* PCL1606, higher attraction was shown to glucose and L-glutamate (1 mM) as well as to the other individual compounds tested. This attraction to these compounds, together with the swim plate assay, suggested that they could be used as carbon sources, which could also be considered an advantage to the microorganism, as previously reported [43].

Interestingly, the use of the capillary assay also allowed for the confirmation of raw and concentrated avocado exudates as very powerful attractants for PCL1606, as indicated by RCI values of 12.5 for raw exudates or 25 for concentrated exudates. Attraction for *R. necatrix* exudates was also observed but with lower RCI values than avocado roots, supporting that chemotaxis could be considered one of the first steps for PCL1606 in initiating interaction with the avocado root and the fungi, which could lead to biocontrol activity, as previously observed [7]. In this sense, it has been described previously that chemotaxis could be considered the first step in root colonization [1,15].

Finally, the use of PCL1606 derivative strains impaired in motility or chemotaxis did not lead to a complete lack of bio-

control, but statistically lower protection could be observed when those strains colonized the roots. In fact, plant protection was reduced, but there was significant protection against *R. necatrix* that was better than with the non-bacterized control. This finding could be due to the PCL1606-*cheA* and PCL1606-*flgK* derivative mutants being inoculated directly on the root for the biocontrol experiments [10]. Thus, the bacterial counts at the end of the experiment did not provide relevant conclusions for colonization behaviour, the involvement of which in plant interaction has been reported previously [1,5,15]. However, a stable population of derivative mutants was reported on avocado roots after 21 days post-inoculation. The wild-type strain and the derivative mutants attained values of approximately 1–2 × 10^6 cfu/g of root, corresponding to the persistence values for PCL1606 on avocado roots after several weeks [19]. That *cheA* and *flgK* mutants still established stable populations on plant roots has been previously described [4,13], suggesting additional adhesive molecules on their cell surfaces to promote cell-root interaction [27]. Interestingly, these strains still produced the antifungal antibiotic HPR, which is considered the primary factor responsible for antagonism and biocontrol [8], and it could help to maintain some biocontrol activity.

Thus, the lack of protection by the mutants PCL1606-*cheA* and PCL1606-*flgK* could result in a delay of the earlier interactions in response to environmental signals (e.g., root and fungal exudates), resulting in a decrease in biocontrol ability but not a complete lack of it. This hypothesis was also supported by the crucial role of the attraction mediated by the plant root and fungal exudates, as well as for individual compounds commonly present in them, for such multitrophic interactions [5].

This study demonstrated that *P. chlororaphis* PCL1606 can detect the compounds exudated by avocado roots and *R. necatrix*. Thus, PCL1606 could have the capacity to detect and move through soil particles towards a gradient of the avocado exudates, as well as to *R. necatrix* exudates, which would result in a more efficient microbe-substrate interaction. For these reasons, motility and chemotaxis could be considered the first steps for biocontrol activity of *P. chlororaphis* PCL1606.

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