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Role of extracellular matrix components in the formation of biofilms and their contribution to the biocontrol activity of *Pseudomonas chlororaphis* PCL1606

Heredia-Ponce, Zaira ; Gutiérrez-Barranquero, José Antonio ; Purtschert-Montenegro, Gabriela ; Eberl, Leo ; de Vicente, Antonio ; Cazorla, Francisco M

Abstract: *Pseudomonas chlororaphis* PCL1606 (PcPCL1606) displays plant-colonizing features and exhibits antagonistic traits against soil-borne phytopathogenic fungi. Biofilm formation could be relevant for the PcPCL1606 lifestyle, and in this study the role of some putative extracellular matrix components (EMC; Fap-like fibre, alginate and Psl-like polysaccharides) in the biofilm architecture and biocontrol activity of this bacterium were determined. EMC such as the Fap-like fibre and alginate polysaccharide play secondary roles in biofilm formation in PcPCL1606, because they are not fundamental to its biofilm architecture in flow cell chamber, but synergistically they have shown to favour bacterial competition during biofilm formation. Conversely, studies on Psl-like polysaccharide have revealed that it may contain mannose, and that it is strongly involved in the PcPCL1606 biofilm architecture and niche competition. Furthermore, the Fap-like fibre and Psl-like exopolysaccharide play roles in early surface attachment and contribute to biocontrol activity against the white root rot disease caused by *Rosellinia necatrix* in avocado plants. These results constitute the first report regarding the study of the extracellular matrix of the PcPCL1606 strain and highlight the importance of a putative Fap-like fibre and Psl-like exopolysaccharide produced by PcPCL1606 in the biofilm formation process and interactions with the host plant root.

DOI: <https://doi.org/10.1111/1462-2920.15355>

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ZORA URL: <https://doi.org/10.5167/uzh-196574>

Journal Article

Accepted Version

Originally published at:

Heredia-Ponce, Zaira; Gutiérrez-Barranquero, José Antonio; Purtschert-Montenegro, Gabriela; Eberl, Leo; de Vicente, Antonio; Cazorla, Francisco M (2021). Role of extracellular matrix components in the formation of biofilms and their contribution to the biocontrol activity of *Pseudomonas chlororaphis* PCL1606. *Environmental Microbiology*, 23(4):2086-2101.

DOI: <https://doi.org/10.1111/1462-2920.15355>

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Journal:	<i>Environmental Microbiology and Environmental Microbiology Reports</i>
Manuscript ID	EMI-2020-1751.R1
Journal:	Environmental Microbiology
Manuscript Type:	EMI - Research article
Date Submitted by the Author:	09-Dec-2020
Complete List of Authors:	Heredia Ponce , Zaira ; University of Malaga, Microbiología Gutiérrez Barranquero, José Antonio; University of Malaga, Microbiología Purtschert Montenegro, Gabriela; University of Zurich, Dep. of Microbiology Eberl, Leo; University of Zurich, Dept. of Microbiology; de Vicente, Antonio; University of Malaga, Microbiología Cazorla, Francisco M.; University of Malaga, Microbiología
Keywords:	biofilm biology, adhesion, microbial ecology, bacteria, fungi

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Role of extracellular matrix components in the formation of biofilms and their contribution to the biocontrol activity of *Pseudomonas chlororaphis* PCL1606

Heredia-Ponce, Zaira¹; Gutiérrez-Barranquero, José Antonio¹; Purtschert-Montenegro, Gabriela²; Eberl, Leo²; de Vicente, Antonio¹; Cazorla, Francisco M.¹

¹Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora” (IHSM-UMA-CSIC) - Departamento de Microbiología, Universidad de Málaga, Bulevar Louis Pasteur, 31 (Campus Universitario de Teatinos), 29071, Málaga, Spain.

²Department of Plant and Microbial Biology. University of Zurich. Zollikerstrasse 107. CH-8008, Zurich, Switzerland.

Corresponding author: Francisco M. Cazorla; email: cazorla@uma.es; Telephone number: [+34 952137587](tel:+34952137587); address: Departamento de Microbiología de la Universidad de Málaga, Bulevar Louis Pasteur, 31 (Campus Universitario de Teatinos), 29071, Málaga (Spain).

Running title: biological role of the EMC of *P. chlororaphis*

Originality-significance statement:

This work is important since it constitutes the first study of the extracellular matrix of the biocontrol agent *P. chlororaphis*. Psl polysaccharide has only been studied in *Pseudomonas aeruginosa* and *Pseudomonas syringae*, in which it plays important roles during biofilm formation. Unprecedented, this work describes the role of a Psl-like exopolysaccharide in the biofilm architecture of a plant-beneficial bacterium. Furthermore, the contribution of a putative Fap-like fibre and Psl-like exopolysaccharide to the biocontrol activity of a strain of *P. chlororaphis* is described for the first time.

Summary:

Pseudomonas chlororaphis PCL1606 (PcPCL1606) displays plant-colonizing features and exhibits antagonistic traits against soil-borne phytopathogenic fungi. Biofilm formation could be relevant for the PcPCL1606 lifestyle, and in this study the role of some putative extracellular matrix components (EMC; Fap-like fibre, alginate and Psl-like polysaccharides) in the biofilm architecture and biocontrol activity of this bacterium were determined. EMC such as the Fap-like fibre and alginate polysaccharide play secondary roles in biofilm formation in PcPCL1606, because they are not fundamental to its biofilm architecture in flow-cell chamber, but synergistically they have shown to favour bacterial competition during biofilm formation. Conversely, studies on Psl-like polysaccharide have revealed that it may

contain mannose, and that it is strongly involved in the PcPCL1606 biofilm architecture and niche competition. Furthermore, the Fap-like fibre and Psl-like exopolysaccharide play roles in early surface attachment and contribute to biocontrol activity against the white root rot disease caused by *Rosellinia necatrix* in avocado plants. These results constitute the first report regarding the study of the extracellular matrix of the PcPCL1606 strain and highlight the importance of a putative Fap-like fibre and Psl-like exopolysaccharide produced by PcPCL1606 in the biofilm formation process and interactions with the host plant root.

Introduction:

Pseudomonas chlororaphis is a common inhabitant of the root environment that belongs to the *Pseudomonas fluorescens* complex (Garrido-Sanz *et al.*, 2017), one of the most diverse bacterial groups within the *Pseudomonas* genus. *P. chlororaphis* typically possesses plant-colonizing activities and exhibits antagonistic traits against soil-borne fungal pathogens (Arrebola *et al.*, 2019). In particular, the *P. chlororaphis* PCL1606 strain (PcPCL1606), isolated from the avocado rhizosphere, has protective features against the soil-borne phytopathogenic fungi *Rosellinia necatrix* and *Fusarium oxysporum*, which produce white root rot disease in avocado and foot and root rot in tomato, respectively (Cazorla *et al.*, 2006; González-Sánchez *et al.*, 2010; Calderón *et al.*, 2015). PcPCL1606 biocontrol activity is mediated by the action of antimicrobial compounds such as proteases and lipases, but mainly production of the compound 2-hexyl 5-propyl resorcinol (HPR), as a direct correlation between the ability to produce this metabolite and a strong inhibition activity against *R. necatrix* and *F. oxysporum* have been observed (Cazorla *et al.*, 2006; Calderón *et al.*, 2013).

The antifungal compound HPR is also involved in the multitrophic interaction avocado root-*R. necatrix*-PcPCL1606 (Calderón *et al.*, 2013, 2014), as well as in biofilm formation and root colonization (Calderón *et al.*, 2014, 2019). Biofilms are accretions of matrix-enclosed microorganisms that adhere to surfaces of biological or non-biological nature. Rhizosphere colonization is favored by the production of an extracellular matrix (Lasík *et al.*, 1989; Matthysse *et al.*, 1998; Lugtenberg *et al.*, 2001; Danhorn & Fuqua *et al.*, 2007; De Weert *et al.*, 2007), but the role that PcPCL1606 extracellular matrix components could play in biofilm formation and the lifestyle of this bacterium remain unknown. The formation of biofilms is a 3-stage process that includes the following steps: 1) attachment to a surface, 2) aggregation of cells, growth and maturation and 3) dispersion (Hall-Stoodley *et al.*, 2004). The first stage consists of adhesion to a surface, and proteinaceous structures such as bacterial adhesins and appendages are fundamental actors in this process. Regarding the protein component of the

65 biofilm matrix of *Pseudomonas*, amyloid-like structures have been gaining importance during
 66 the last decade (Dueholm *et al.*, 2010, 2013; Taglialegna *et al.*, 2016). In particular, Fap
 67 fimbriae could have environmental relevance due to their potential role in plant root
 68 colonization (Dueholm *et al.*, 2013; Rouse *et al.*, 2018). Exopolysaccharides are the main
 69 matrix components necessary for cell aggregation and biofilm maturation. In general,
 70 *Pseudomonas* spp. produces several polysaccharide biofilm matrix molecules, including
 71 alginate, levan, cellulose and Psl polysaccharide (Fuchs *et al.*, 1956; Conti *et al.*, 1994;
 72 Bianciotto *et al.*, 2001; Spiers *et al.*, 2003, 2005; Friedman & Kolter, 2004; Jackson *et al.*,
 73 2004; Matsukawa & Greenberg, 2004). Alginate is composed of β -1,4 linked monomers of L-
 74 glucuronic and D-mannuronic acids (Evans & Linker, 1973), and although it is not
 75 fundamental for the constitution of biofilms in *Pseudomonas aeruginosa* and *Pseudomonas*
 76 *syringae* (Wozniak *et al.*, 2003; Laue *et al.*, 2006; McIntyre-Smith *et al.*, 2010; Heredia-
 77 Ponce *et al.*, 2020), its participation in protection against stressors and biofilm structure have
 78 been proven in these species (Schnider-Keel *et al.*, 2001; Hentzer *et al.*, 2001; Pier *et al.*,
 79 2001; Leid *et al.*, 2005; Heredia-Ponce *et al.*, 2020). Levan polysaccharide, a polymer of β -
 80 2,6 linked fructose, has been mainly studied in *P. syringae*, where the involvement in biofilm
 81 architecture does not seem to be relevant (Laue *et al.*, 2006). Rather, levan accumulation in
 82 cell free areas of biofilms suggests a role as a storage molecule (Laue *et al.*, 2006). Cellulose
 83 is a glucose polymer with β -1,4 glycosidic linkages, which is considered to be an important
 84 biofilm matrix molecule in many environmental pseudomonads (Spiers *et al.*, 2002, 2003,
 85 2005; Mann & Wozniak *et al.*, 2012). Although several biosynthesis and regulation
 86 mechanisms have been described for bacterial cellulose, a common role of this component is
 87 to facilitate the establishment of efficient host-bacteria interactions (Augimeri *et al.*, 2015).
 88 The described Psl polysaccharide, composed of D-mannose, D-glucose and L-rhamnose
 89 (Friedman & Kolter, 2004; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Byrd *et al.*,
 90 2009), has been mainly studied in *P. aeruginosa* (Ma *et al.*, 2009; Zhao *et al.*, 2014; Wang *et al.*,
 91 *et al.*, 2013, 2014; Yang *et al.*, 2018). Recently, its involvement in the biofilm architecture,
 92 virulence and motility have also been described in the plant-associated bacterium *P. syringae*
 93 *pv. syringae* (Heredia-Ponce *et al.*, 2020). Although the presence of the *psl*-like gene cluster
 94 has been reported in some environmentally relevant pseudomonads (Records & Gross, 2010;
 95 Mann & Wozniak *et al.*, 2012), including the biocontrol agent *P. fluorescens* Pf5, the
 96 biological participation of this polysaccharide in beneficial plant-bacteria interactions remains
 97 undetermined.

In this study, we identified a genomic region that putatively encodes a Fap-like amyloid fibre and two genomic regions that putatively encode alginate and Psl-like exopolysaccharides in the PcPCL1606 strain. Since biofilm formation could play a relevant role in the PcPCL1606 lifestyle, research regarding these biofilm components is explored in this work. Thus, the main aim of this work was to study the potential involvement of the Fap-like, alginate and Psl-like-encoding regions in biofilm formation and the lifestyle of PcPCL1606 during its interactions with the host plant root.

Results:

Bioinformatics analysis of the *P. chlororaphis* PCL1606 genome revealed the presence of gene clusters encoding a putative Fap fibre, an alginate and Psl-like exopolysaccharides.

The presence of a Fap-like-, alginate- and Psl-like-encoding clusters has been reported in the PcPCL1606 strain for the first time. An *in-silico* analysis was performed to identify and characterize the genomic regions that might encode these components in PcPCL1606. Using the *P. aeruginosa* PAO1 (PAO1) PA1951-PA1956 genomic region as a template, which encodes the Fap system, the PCL1606_RS15320-PCL1606_RS15345 genomic region of PcPCL1606 was found to show similarity (Supplementary Figure S1A). These similarities were even higher when the *fap* clusters of the *P. fluorescens* UK4 strain (PfUK4), the first strain in which the *fap* operon was described (Dueholm *et al.*, 2010), and PcPCL1606 were compared (Supplementary Figure S1B). The FapC protein constitutes the main protein of the fibre and contains three proteinic repeat motifs (R1-R3; Dueholm *et al.*, 2010). Clustal alignments were performed between each of the three proteinic repeat motifs (R1-R3) present in the FapC protein of PfUK4 and the putative FapC protein of the PcPCL1606 strain (Supplementary Figure S1C). These alignments revealed high conservation patterns among the amino acids present in those regions (Supplementary Figure S1C).

Regarding exopolysaccharide production clusters, the PFLU0300-PFLU0309 genomic region of the *P. fluorescens* SBW25 (SBW25) strain and the Psyrmg_RS20465–Psyrmg_RS20505 genomic region of the phytopathogen *P. syringae* pv. *syringae* UMAF0158 (PssUMAF0158) strain, which correspond to cellulose operon sequences, were not orthologous to any region located in the genome of the PcPCL1606 strain. The PCL1606_RS24735-PCL1606_RS24790 genomic region of PcPCL1606 was orthologous to the PA3540-PA3551 genomic region of PAO1, which encodes proteins of the alginate operon (Supplementary Figure S2). Additionally, the *psl* operon sequence of PAO1 and *psl*-like gene cluster of PssUMAF0158, which correspond to the PA2231-PA2245 and Psyrmg_RS06720-Psyrmg_RS06770 genomic

regions, respectively, allowed us to identify the orthologous PCL1606_RS04710-PCL1606_RS04765 genomic region in PcPCL1606 (Supplementary Figure S3). As previously described in PssUMAF0158 (Heredia-Ponce *et al.*, 2020), the gene that encodes the PslO protein is missing and the PslN-like (PCL1606_RS12970) protein seem to be encoded somewhere else in the chromosome outside the *psl*-like gene cluster (Supplementary Tables S1A-C). The PslM-like (PCL1606_RS04265) protein, which is missing in PssUMAF0158, seems to be encoded somewhere else in the chromosome outside the *psl*-like gene cluster in PcPCL1606 (Supplementary Tables S1A-C). Furthermore, the PAO1 PslL acyltransferase has not been associated with any protein encoded in the PcPCL1606 genome, as it was previously reported in PssUMAF0158 (Heredia-Ponce *et al.*, 2020). However, the PCL1606_RS04715 gene, which is located within the *psl*-like gene cluster, encodes a putative acetyltransferase that might be performing a related function (Supplementary Table S1B). This acetyltransferase shares approximately 60% identity with an acetyltransferase of PssUMAF0158 encoded by an equivalent gene within the *psl*-like gene cluster. Most of the protein domains encoded within the *psl* cluster of PAO1 and *psl*-like gene cluster of PssUMAF0158 are conserved in PcPCL1606 (Supplementary Tables S1A-C).

Phylogenetic analysis revealed that the *psl*-like gene cluster has a random distribution within the *P. fluorescens* complex. To elucidate the phylogeny of the *psl*-like gene cluster within the *P. fluorescens* complex, a total of 50 strains belonging to the different phylogenetic groups included within the *P. fluorescens* complex and isolated from different habitats were selected and used for the analysis (Supplementary Table S2). The recently described *psl*-like gene cluster of PssUMAF0158 was also included in the analysis. The combined nucleotide sequences of the *gyrB* and *rpoD* housekeeping genes clearly supported the phylogenetic distribution in the different phylogroups included in the *P. fluorescens* complex (Garrido-Sanz *et al.*, 2016). The *psl*-like gene cluster was absent in the strains included in Garrido-Sanz *et al.*, 2017 as belonging to the *corrugata*, *jessenii* and *koreensis* phylogroups. Of all the strains included in the *mandelii* phylogroup, only *Pseudomonas* GM21 presented the *psl*-like gene cluster. Except for the *chlororaphis* phylogroup, in which the *psl*-like gene cluster was present in all the strains tested (Supplementary Table S3), the rest of phylogroups contained strains that presented the cluster and strains that did not. The phylogenetic distribution of the strains selected based on the housekeeping genes (Figure 1A) did not correlate with their phylogenetic distribution based on the *psl*-like gene cluster (Figure 1B).

The putative Fap fibre and a Psl-like exopolysaccharide of *P. chlororaphis* PCL1606 are involved in the initial attachment to abiotic surfaces. To investigate the role of the extracellular matrix components under study at the initial stages of PcPCL1606 biofilm formation, short-term adhesion experiments to polystyrene microtiter wells and Crystal violet staining were performed. The results showed that both the Fap-like fibre and the Psl-like exopolysaccharide, but not alginate, were involved in the initial surface attachment (Figure 2). The impaired attachment values were not accumulative for the $\Delta psIE$ and $\Delta fapBC$ mutants, as we observed up to 60% reduction of the attachment levels for the single, double and triple mutants that included the *psIE* gene deletion and up to a 50% reduction for the single and double mutants that included the *fapBC* genes deletion. The complemented strains showed a restoration of the mutant phenotype to wild-type levels.

Production of the Psl-like exopolysaccharide is essential for biofilm formation in *P. chlororaphis* PCL1606. To investigate the roles of the Fap-like fibre, alginate and Psl-like exopolysaccharides in the biofilm architecture of the PcPCL1606 strain, flow-cell chamber experiments were performed, and the biofilm architecture of the wild-type strain was compared to that of its derived extracellular matrix mutants (Figure 3). Confocal laser scanning microscopy (CLSM) and IMARIS software reconstruction allowed us to display representative 3D images of the formed biofilms in the flow-cells. After 48 h, the $\Delta fapBC$ and $\Delta alg8$ single mutants, as well as the $\Delta fapBC, alg8$ double mutant, formed biofilms that were undistinguishable from the wild-type strain (Figure 3A). The biofilms formed by these strains were characterized by a multilayer of gathered cells that covered the flow-cell chamber surface, and the presence of uncolonized space among the aggregating cells were barely noticeable (Figure 3A). In contrast, the biofilms formed by the $\Delta psIE$ mutant consisted of a monolayer of loosely aggregated cells, where the amount of uncolonized space was more perceptible (Figure 3A). The double and triple mutants that included the *psIE* gene deletion showed the same biofilm phenotype as the $\Delta psIE$ single mutant (Figure 3A). The area and volume of the biofilms formed by the $\Delta fapBC$ and $\Delta alg8$ single mutants, as well as the $\Delta fapBC, alg8$ double mutant, were not altered compared to the wild-type strain (Figure 3B and 3C). All the mutants that contained the *psIE* gene deletion showed an impaired biofilm phenotype, which was characterized by a decrease in surface coverage and volume occupied (Figure 3B and 3C).

The Psl-like exopolysaccharide contains mannose and is allocated to the extracellular matrix of *P. chlororaphis* PCL1606. The Psl polysaccharide of the *P. aeruginosa* PAO1

strain is primarily composed of mannose (Ma *et al.*, 2007; Byrd *et al.*, 2009). Thus, to illustrate the presence of Psl-like exopolysaccharide in the extracellular matrix of PcPCL1606 biofilms, lectin staining of 48 h-old biofilms formed by the wild-type strain and its derived Psl-like exopolysaccharide mutant was performed using FITC-labelled *Musa paradisiaca* lectin (BanLec), which binds to mannose residues (Figure 4). The biofilm formed by the PcPCL1606 wild-type strain was able to bind lectin, while the *ΔpslE* mutant strain was not.

The putative Fap fibre, alginate and Psl-like exopolysaccharides provide a competitive advantage during biofilm formation in *P. chlororaphis* PCL1606. To investigate whether the extracellular matrix components under study participate in bacterial competition and niche colonization features displayed by PcPCL1606, competition experiments in flow-cell chambers were performed using mixed biofilms containing the mCherry-tagged wild-type bacterium and the corresponding GFP-tagged extracellular matrix mutant (Figure 5A). The results were analysed as the percentage of colonized space (area and volume) occupied by the wild-type strain and each mutant (Figures 5B and 5C). Competition assays between the *Δalg8* or *ΔfapBC* single mutants and the wild-type strain revealed equivalent fitness in niche competition due to the biofilm formed by the wild-type and each mutant occupied approximately 50% of the area and volume of the biofilm when both were coinoculated (Figure 5B and 5C). However, the *Δalg8, fapBC* double mutant was significantly affected, as within the mixed biofilm, the wild-type strain occupied significantly higher area and volume than the *Δalg8, fapBC* strain. In addition, the *ΔpslE* single mutant, as well as the double and triple mutants containing the *pslE* gene deletion, were severely impaired in niche colonization compared with the wild-type strain (Figure 5A). Thus, *pslE* gene deletion abrogated the ability of the PcPCL1606 strain to compete for biofilm formation with the wild-type equivalent, as the surface coverage and volume occupied by the mutants within the mixed biofilm were markedly reduced (Figure 5B and 5C).

The Fap-like fibre and the Psl-like exopolysaccharide contribute to the biocontrol activity of the *P. chlororaphis* PCL1606 strain. Biocontrol assays against the avocado white root rot disease caused by *R. necatrix* were conducted on the *ΔpslE* and *ΔfapBC* single mutants because the putative Fap fibre and Psl-like exopolysaccharide showed the strongest effects in biofilm-related phenotypes (Figure 2 and 3)., and Aerial symptoms were evaluated using the previously described disease scale (Cazorla *et al.*, 2006). ~~Experiments in the *R. necatrix*-avocado system were performed using the *ΔfapBC* and *ΔpslE* mutants, as they showed the strongest effects on biofilm-related phenotypes.~~ When no bacteria were applied to

the avocado roots, the plants infected with *R. necatrix* reached a disease index value of approximately 76% on day 28 post-inoculation (Figure 6A). However, when the plants were previously inoculated with the PcPCL1606 wild-type strain, the disease index reached approximately 48% on the same day (Figure 6A). The plants that had been inoculated with the *ΔfapBC* and *ΔpslE* mutant strains prior to *R. necatrix* inoculation reached disease indices that were similar to those observed when the plants were not inoculated and subsequently higher than those observed when the plants were inoculated with the biocontrol wild-type strain (Figure 6A). The area under the disease progress curve (AUDPC) showed that when the plants were inoculated with the PcPCL1606 wild-type strain, the disease severity was reduced by approximately 50% compared with those that were not previously inoculated (Figure 6B). Instead, when the plants were inoculated with the *ΔfapBC* and *ΔpslE* mutants, disease development appeared similar to that observed in plants that were not previously inoculated (Figure 6B). In summary, biocontrol activity was impaired in the *ΔfapBC* and *ΔpslE* mutants, and it was restored to wild-type levels in the *fapBC* and *pslEF* complemented strains (Figure 6B). In addition, CLSM experiments revealed that the *ΔfapBC* and *ΔpslE* mutants showed alterations in their early attachment to avocado roots compared with the wild-type strain (Figure 6C). The wild-type strain showed wider avocado root attachment than the *ΔfapBC* and *ΔpslE* mutants, with both individual and clumps of cells adhered to the avocado root cells. However, the *ΔfapBC* mutant showed less attachment of the root, the pattern of which was characterized by dispersed cell adhesion located among root cells. Finally, the *ΔpslE* mutant also showed less attachment to the roots, exhibiting an irregular pattern.

Discussion:

Biofilm formation plays an advantageous, if not essential, role in plant-bacteria interactions in many environmentally relevant *Pseudomonas* (Morris & Monier, 2003; Danhorn & Fuqua, 2007). Plant-associated bacteria can interact with the aerial parts, the rhizosphere or even the vascular system of the plant host using a great diversity of extracellular polymeric substances, including exopolysaccharides, proteins and DNA (Ramey *et al.*, 2004). Even though some *P. chlororaphis* strains have been reported as biofilm formers (Maddula *et al.* 2006; Poritsanos *et al.* 2006; Kim *et al.* 2014; Calderón *et al.*, 2019), little attention has been paid to the study of their extracellular matrix components. The *P. chlororaphis* PCL1606 strain (PcPCL1606) is a biofilm-forming bacterium with root-colonization and biocontrol activities (Cazorla *et al.*, 2006; Calderón *et al.* 2019; Arrebola *et al.*, 2019). The PcPCL1606 extracellular matrix could play a relevant role in these processes, and thereby, based on its lifestyle, the involvement of

some putative extracellular matrix components in biofilm formation ability and biocontrol activity of this strain were explored in this work.

In this study, three genomic clusters were identified in the PcPCL1606 strain, which putatively encode a Fap amyloid-like fibre and two exopolysaccharides, alginate and Psl polysaccharide. These genomic regions might be involved in biofilm formation, as their relevance in other *Pseudomonas* has been previously revealed (Bianciotto *et al.*, 2001; Friedman & Kolter, 2004; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Franklin *et al.*, 2011; Dueholm *et al.*, 2013; Marshall *et al.*, 2019; Heredia-Ponce *et al.*, 2020). The genomic regions encoding the Fap fibre and alginate exopolysaccharide have been identified in several species of *Pseudomonas* (Conti *et al.*, 1994; Stapper *et al.*, 2004; Laue *et al.*, 2006; Rouse *et al.*, 2018; Marshall *et al.*, 2019). However, the genomic regions encoding a Psl-like exopolysaccharide have only been identified in *P. aeruginosa*, some strains of *P. syringae*, *P. mendocina* ymp and *P. fluorescens* Pf5 (Mann & Wozniak, 2012; Heredia-Ponce *et al.*, 2020). Regardless, the biological role of Psl exopolysaccharide in beneficial plant-bacteria interactions is unknown to date. The results obtained in the phylogenetic analysis (Figure 1) suggest that the distribution of the *psl*-like gene cluster within *P. fluorescens* complex is promiscuous. Additionally, the presence of the *psl*-like gene cluster in isolates of different sources (Supplementary Table S2) further suggests that the ability to produce this exopolysaccharide may not be habitat-dependent, but a more general feature of biofilm formation, and that this gene cluster could have been acquired by horizontal gene transfer. The *psl*-like cluster is widely distributed within the *chlororaphis* phylogroup (Supplementary Table S3), which may explain why relevant roles in biofilm formation and related aspects have been observed in the PcPCL1606 strain in this study.

The initial stage in the biofilm formation process consists of attachment to a surface (Hall-Stoodley *et al.*, 2004). Proteinaceous structures, such as bacterial adhesins and appendages, play fundamental roles in this process (Berne *et al.*, 2015). Although exopolysaccharides were thought to be mainly required for later architectural development of the biofilms (Sutherland, 1999), implications in the initial attachment to the surfaces of roots have also been reported in several species (Wheatley *et al.*, 2018). In agreement with previous studies (Colvin *et al.*, 2012; Berne *et al.*, 2015), the Fap-like fibre and Psl-like exopolysaccharide of the PcPCL1606 strain were found to be involved in the initial attachment to abiotic surfaces (Figure 2). The finding that alginate did not play an important role in PcPCL1606 during initial surface attachment (Figure 2) was also consistent with other studies performed in *P. aeruginosa*, in

which this polysaccharide was not truly required for the early steps of biofilm formation (Wozniak *et al.*, 2003; McIntyre-Smith *et al.*, 2010).

Regarding later stages of biofilm development, the Psl-like exopolysaccharide was proven to be an essential component of the biofilm architecture of the PcPCL1606 strain (Figure 3). This result was in line with previous studies, in which the Psl polysaccharide was reported to be important for adhesion and maintaining the biofilm structure post-attachment (Friedman & Kolter, 2004; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Ma *et al.*, 2006; Heredia-Ponce *et al.* 2020). After 48 h of incubation, the biofilm phenotype of the PcPCL1606 *ApsIE* strain resembled that of the PAO1 Psl-like mutant (Jackson *et al.*, 2004; Ghafoor *et al.*, 2011), consisting of a monolayer of cells (Figure 3A and 3C), which suggests that the Psl-like exopolysaccharide could be decisive for the constitution of the three-dimensional architecture of the PcPCL1606 biofilm. In *P. aeruginosa* PAO1, the most recent study determined that Psl polysaccharide is composed of a pentasaccharide subunit containing D-mannose, D-glucose and L-rhamnose, at approximate ratios of 3:1:1 (Byrd *et al.*, 2009). However, this result conflicts with previous reports, in which the presence of galactose in the Psl structure was also reported (Ma *et al.*, 2007). In any case, mannose is described as the main monosaccharide of the polymer. BanLec staining of the Psl-like exopolysaccharide of the PcPCL1606 strain suggested that it also contained mannose (Figure 4), which is in agreement with prior studies on *P. aeruginosa* and suggests that these polysaccharides might actually be similar in composition. The alginate mutants of *P. aeruginosa*, *P. syringae* and *P. fluorescens* retained the ability to form biofilms compared to their respective wild-types, but their biofilm architectures were different (Wozniak *et al.*, 2003; Stapper *et al.*, 2004; Laue *et al.*, 2006; Ghafoor *et al.*, 2011; Noirot-Gros *et al.*, 2019; Heredia-Ponce *et al.*, 2020). However, no impairment in biofilm architecture was observed in the *Aalg8* mutant strain of PcPCL1606 (Figure 3). The presence of the *fap* operon has been reported in several *Pseudomonas* species (Mann & Wozniak *et al.*, 2012; Dueholm *et al.*, 2013), but its role in biofilm formation has been considered as secondary, because the PAO1 Fap mutant formed biofilms to the same extent as the wild-type strain (Dueholm *et al.*, 2013). In line with the published data, and despite the differences observed in early adhesion in the *AfapBC* mutant (Figure 2 and 6C), similar surface coverage and volumes values of the biofilms formed by the PcPCL1606 wild-type strain and *AfapBC* mutant have been observed (Figure 3), leading to a similar conclusion. Thus, similar to *P. aeruginosa* (Dueholm *et al.*, 2013), the role of the PcPCL1606 Fap-like fibre seems to be more directed towards initial attachment of cells to surfaces, as the early adhesion deficiencies of the *AfapBC* mutant (4 h) were not observed at later time periods (6

329 and 8 h; Supplementary Figure S4) and the developed biofilm architecture of the mutant was
 330 indistinguishable from that of the wild-type (Figure 3).

331 The ability to produce Psl-like polysaccharide provided a selective advantage to synthesizing
 332 bacteria in competition for biofilm formation (Figure 5). This result had been previously
 333 observed in *P. aeruginosa*, in which the Δ *psl* mutant of PAO1 strain could not be rescued by
 334 the parental strain when both were co-cultured (Jackson *et al.*, 2004; Irie *et al.*, 2016).
 335 Interestingly, these competition experiments suggest that Psl is acting as a private good in
 336 PcPCL1606, as it has been previously reported in *P. aeruginosa* (Irie *et al.*, 2016). Instead, the
 337 Fap-like fibre and the alginate exopolysaccharide were shown to play secondary roles in
 338 biofilm formation of the PcPCL1606 strain, as the biofilms formed by the mutant strains
 339 showed surface coverage and volume values that were undistinguishable from those of the
 340 wild-type (Figure 3), but together they contributed synergistically to biofilm architecture by
 341 providing colonization advantages during biofilm formation (Figure 5). This result correlates
 342 with the impaired phenotype in early attachment observed in the Δ *fapBC*,*alg8* double mutant
 343 (Figure 2). Cooperation between different extracellular matrix components is not uncommon,
 344 as some of them have been described to only function synergistically during biofilm
 345 formation (Saldaña *et al.*, 2009; Ostrowski *et al.*, 2011; Fong *et al.*, 2015).

346 Colonization of the roots is one of the first steps in the establishment of beneficial plant-
 347 bacteria interactions (De Weert & Bloemberg, 2007). Since PcPCL1606 is a biocontrol agent
 348 of plant-fungal diseases, it was essential to ascertain the contribution of the Fap-like fibrils
 349 and the Psl-like exopolysaccharide, which showed the strongest effects in biofilm related
 350 phenotypes, in disease control. Both biofilm components were found to be relevant in white
 351 root rot disease suppression (Figures 6A and 6B). The putative roles of Psl polysaccharide in
 352 the surface colonization of *P. aeruginosa* (Zhao *et al.*, 2014) and *P. syringae* (Heredia-Ponce
 353 *et al.*, 2020), as well as the presence of a *psl*-like gene cluster in some pseudomonads that
 354 usually colonize the soil and roots of plants (Supplementary Table S2), revealed the potential
 355 involvement of the PcPCL1606 Psl-like exopolysaccharide in the interaction with the plant
 356 root. There is evidence suggesting that the Fap system could also play a role in the interaction
 357 with the plant root (Dueholm *et al.*, 2013; Rouse *et al.*, 2018). The obtained results revealed a
 358 clear influence of the Fap-like fibre and the Psl-like exopolysaccharide in PcPCL1606
 359 biocontrol ability (Figures 6A and 6B). Thus, the impairment in both initial abiotic and biotic
 360 surface attachment observed in the Δ *fapBC* and Δ *pslE* mutants (Figure 2 and 6C), as well as
 361 the contribution of the Psl-like exopolysaccharide to the PcPCL1606 overall biofilm

architecture (Figures 3 and 5), could all indicate that inefficient interaction with the plant root by the mutants might actually lead to an impairment of disease suppression, probably by allowing a better access of the fungus to the plant root or by providing an ineffective delivery system of antifungal metabolites, as previously suggested (Chin-A-Woeng *et al.*, 2007). Thus, effective colonization of the rhizosphere appears as a prerequisite for the efficient disease control of PcPCL1606 ~~by preventing pathogen access to the root~~, in agreement with former studies performed in biocontrol agents (Bloemberg & Lugtenberg, 2001; Lugtenberg & Kamilova, 2009).

In conclusion, our work constitutes the first report regarding the presence of a *psl*-like gene cluster within the *P. chlororaphis* group, its role in biofilm formation and influence on the biocontrol lifestyle in the plant-beneficial bacterium PcPCL1606. Furthermore, the Fap-type system and alginate exopolysaccharide of PcPCL1606, although not fundamental for biofilm formation, seem to cooperatively contribute to biofilm formation in this bacterium by proving relevance during competitive colonization in biofilm formation experiments. Additionally, this is the first time that the role in early surface interactions and the biocontrol lifestyle has been demonstrated for a Fap-like fibre, in a manner similar to that of the Psl-like exopolysaccharide. These results contribute to a better understanding of the biofilm matrix of the PcPCL1606 biocontrol strain and its involvement in the multitrophic interactions that take place during the biological control of soil-borne plant pathogens.

Experimental procedures:

• Bacterial strains and growth conditions:

The bacterial strains and plasmids used in this study are listed in Supplementary Table S4. For standard maintenance, PcPCL1606 (Cazorla *et al.*, 2006) and the extracellular matrix mutants were grown in tryptone-peptone-glycerol medium (TPG; Calderón *et al.*, 2014a) at 25°C. *Escherichia coli* was used as a host for the mutation and complementation plasmids and was routinely grown in lysogenic broth (LB) at 37°C. The antibiotics used for the selection of *P. chlororaphis* derivatives were kanamycin 50 mg/L (Km₅₀) and gentamycin 50 mg/L (Gm₅₀). The antibiotics used for the selection of *E. coli* derivatives were kanamycin 50 mg/L (Km₅₀), ampicillin 100 mg/L (Ap₁₀₀) and gentamycin 10 mg/L (Gm₁₀). TPG medium was used for the *in vitro* experiments. Pseudomonas Isolation Agar (PIA; Difco) was used for PcPCL1606 fluorescent tagging by triparental conjugation. Flow-cell chamber experiments were performed using ABC minimal medium (Clark & Maaløe, 1967).

394 • **Bioinformatics analysis:**

395 Nucleotide and protein sequence searches were performed with the *Pseudomonas* Genome
396 Database (<https://www.pseudomonas.com/>) and the National Center for Biotechnology
397 Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>). Putative protein domain
398 searches were carried out using Protein Family Software (PFAM) (<https://pfam.xfam.org/>).

399 • **Construction of bacterial mutants and tagging:**

400 *P. chlororaphis* PCL1606 (PcPCL1606) knockout extracellular matrix mutants were
401 generated according to the protocol described in Zumaquero *et al.*, 2010. Specific primers are
402 listed in Supplementary Table S5. The transformants were selected on TPG medium
403 containing kanamycin. To assess whether each transconjugant integrated the plasmid into the
404 chromosome or engaged in allelic exchange, PCR amplifications of a fragment within the
405 selected gene and southern blot analysis using a fragment within the gene and the Km cassette
406 as probes were performed. To create the doubles and triple mutants, the kanamycin cassette
407 was removed using a modified pFPL2 plasmid, in which the gentamycin cassette present in
408 the pBBR1MCS5 plasmid was amplified and introduced into the pFLP2 plasmid using
409 primers containing the BamHI and XbaI restriction sequences (Supplementary Table S5). The
410 complementation plasmids were constructed using specific primers that included the
411 amplification of the fifty base pairs upstream of the selected gene, to include their own
412 ribosome binding site. For *pslE* gene complementation, both the *pslE* and *pslF* genes were
413 amplified. The resulting amplicons were then cloned into the pBBR1MCS5 plasmid and fully
414 sequenced to discard any possible mutations. Fluorescent tagging was performed using the
415 triparental conjugation protocol described in Lambertsen *et al.*, 2004. Briefly, the *E. coli* S17-
416 1 donor, *E. coli* S17-1 helper and recipient strains were grown overnight at an appropriate
417 temperature (37°C for *E. coli* and 25°C for *Pseudomonas*). Then, the bacterial suspensions
418 were washed (1x) with 0.85% NaCl solution. Subsequently, 200 µl of each donor and helper,
419 and 100 µl of the recipient strain, were mixed, plated in LB-agar and incubated overnight at
420 25°C. The conjugation spots were subsequently resuspended in 0.85% NaCl solution, serial
421 diluted 10-fold and spread on PIA plates to avoid *E. coli* growth. The plates were incubated
422 48 h at 25°C for single fluorescent PcPCL1606 colonies to appear.

423 • **Phylogenetic analysis:**

424 The phylogenetic analyses were performed using combined sequences of the *gyrB* and *rpoD*
425 housekeeping genes, along with the complete nucleotide sequence of the *psl*-like gene

clusters. The *psl*-like gene clusters were identified performing megablast and **discontinuous** **discontiguous** megablast searches in BLASTN with the PcPCL1606 *psl*-like gene cluster as a model (*pslABCDEFGHIJKL*-like genes), as well as the genomes of fifty strains belonging to the different phylogenetic groups included in the *P. fluorescens* complex (Supplementary Table S2). Sequence alignment was performed using ClustalW (Larkin *et al.*, 2007). Phylogenetic trees were constructed using MEGAX software with Jukes Cantor's algorithm and the maximum likelihood (ML) statistical method. The confidence level for the branching points was determined using 1,000 bootstrap replicates. *P. aeruginosa* PAO1 was used as an outgroup.

• **Bacterial attachment assay:**

The attachment assays were conducted as previously described (McCarthy *et al.*, 2014; Gutiérrez-Barranquero *et al.*, 2015), with slight modifications. Five hundred microliters of the PcPCL1606 wild-type strain and its derivative extracellular matrix mutant at an OD of 0.25 a.u. at 600 nm (approx. 10^8 CFU/ml) were inoculated in 24-well microtiter plates. To ensure that there were no differences in bacterial concentration at the time of inoculation, cell counts of the bacterial suspensions were performed. After 4, 6 or 8 h of incubation at 25°C, cultures were removed and washed with distilled water. Five hundred microliters of 0.1% crystal violet (Panreac) as added to each well, followed by an incubation period of 15 min. Then, the crystal violet was removed, and the wells were vigorously washed with tap water. The plates were subsequently dried at 37°C for 2 h. Finally, 500 μ l of 96% EtOH were added to each well and incubated under agitation at room temperature for 15 min. One hundred microliters was then collected from each well and added to a clean 96-well plate for absorbance measurements at 595 nm. Finally, the fold change data were obtained by normalizing the absorbance values relative to the wild-type average of each independent experiment.

• **Flow-cell assays and biofilm visualization:**

To assess differences regarding biofilm formation between the PcPCL1606 wild-type and its knockout extracellular matrix mutants, flow-cell chambers were prepared and assembled as previously described (Christensen *et al.*, 1999). Fresh GFP-tagged wild-type and GFP-tagged extracellular matrix mutants were inoculated as single colonies into 5 ml of TPG tubes and incubated overnight at 200 rpm and 25°C. Then, the cultures were adjusted to a low cell density with 0.85% NaCl solution (OD of 0.01 a.u at 600 nm, which corresponds to 10^6 CFU/ml), and 300 μ l was injected into each channel with a small syringe. After inoculation, the flow remained stopped for 1 h to allow attachment of the cells to the channels. The flow-

cells were supplied with ABC minimal medium (Clark & Maaløe, 1967). Medium flow was maintained at a constant rate of 1.3 ml/min by a Watson-Marlow 205S peristaltic pump and the incubation temperature was 25°C. Microscopic inspection and image acquisition were performed using a confocal laser scanning microscope (CLSM; DM5500Q, Leica) equipped with a 40/1.3 or a 63/1.4 oil objective. Captured images obtained with the Leica Application Suite (Mannheim, Germany) were used to quantify biofilms area and volume values using the IMARIS software package (Bitplane, Switzerland).

- **Lectin staining of Psl-like components:**

The putative presence of a Psl-like polysaccharide within the PcPCL1606 extracellular matrix was assayed using the mCherry-tagged wild-type and the mCherry-tagged *ΔpslE* mutant strain, as well as the FITC-tagged *Musa paradisiaca* lectin (BanLec; Glycomatrix), which binds to mannose residues (Singh *et al.*, 2014). First, the biofilms were grown in flow-cell chambers as mentioned above. BanLec was used at a final concentration of 100 µg/ml, as previously described for lectins (Neu *et al.*, 1999). Forty-eight hours postinoculation, the flow was stopped and 100 µl of a 100 µg/ml lectin solution was gently injected into the chamber, to avoid complete wash out of the biofilms. After 20 min of staining, the flow was restarted to allow the clearance of the unbound protein for 20 min before microscopic observation. Subsequently, the images were recorded and analysed with the Leica Application Suite (Mannheim, Germany) and IMARIS software package (Bitplane, Switzerland). To detect the cells, the 532-nm laser was used for mCherry fluorophore excitation, and emission was monitored at 570-700 nm. The FITC-tagged BanLec was excited with the 488-nm laser, and emission was monitored at 450-500 nm.

- **Competition experiments during biofilm formation:**

The competition experiments during biofilm formation were performed in flow cells using ABC minimal medium (Clark & Maaloe, 1967). The flow-cell system was prepared and assembled as previously described (Christensen *et al.*, 1999). The channels were inoculated with a 1:1 mixture of differentially mCherry-tagged wild-type and GFP-tagged extracellular matrix mutants, previously grown at a low cell density ($OD_{600nm} = 0.01$ a.u., which corresponds to 10^6 CFU/ml). Images were recorded and analysed with the Leica Application Suite (Mannheim, Germany) and IMARIS software package (Bitplane, Switzerland). The 532-nm laser was used for mCherry fluorophore excitation, and emission was monitored at 570-700 nm. The 488-nm laser was used for GFP fluorophore excitation, and emission was monitored at 450-500 nm.

• **Biocontrol assays against avocado white root rot:**

The biocontrol assays against avocado white root rot disease were performed as previously described (Cazorla *et al.*, 2006). The roots of 6-month-old avocado plants obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain) were disinfected with 1% v/v household bleach for 20 min, washed with distilled water and inoculated as previously described (Calderón *et al.*, 2013). For the inoculation process, the roots of the avocado plants were immersed either in a suspension of the bacterial isolate (10^8 CFU/ml) or in sterile TPG medium (controls) for 20 min. Excess bacteria were removed from the roots, and the seedlings were transplanted into pots containing potting soil (Jongkind Grond B.V., Aalsmeer, The Netherlands) and subsequently infected with *Rosellinia necatrix* using eight colonized wheat grains per pot (Freeman *et al.*, 1986). The plants that were not inoculated but were infected served as negative controls for biocontrol activity. Furthermore, the plants that were neither inoculated nor infected served as environmental controls. Two independent experiments, each with at least 7 avocado seedlings per strain, were performed. Aerial symptoms were recorded on a scale from 0 to 3 (from 0 – not affected – to 3 – dead plant). The disease index was calculated using the previously described formula (Cazorla *et al.*, 2006), and to minimize the variability inherent to the experiments with plants, all values were normalized to the wild-type average for each independent experiment.

• **Bacterial adhesion to avocado roots:**

The avocado roots were disinfected in 1% v/v household bleach for 20 min, washed in distilled water and immersed in GFP-tagged bacterial cultures at a final concentration of 10^8 CFU/ml for 20 min, as previously described (Calderón *et al.*, 2013). Then, each root was washed again by immersion in distilled water to remove the free and weakly attached bacteria and transplanted into pots filled with vermiculite. After 4 h of incubation, the plants were removed from the pots, and superficial root samples were randomly collected with a scalpel and placed on glass slides for examination. The fluorescently-labelled bacteria attached to the roots were visualized by confocal laser scanning microscopy (CLSM) using a Zeiss LSM 880 microscope. Three independent experiments, each with three avocado seedlings per strain, were performed.

Acknowledgments:

The authors would like to thank Dr. Aurélien Bailly (University of Zurich) for guidance and assistance with the confocal microscopy, Sandra Tienda (University of Malaga) for the help

and assistance with the experiments with plants and Irene Linares (University of Malaga) for her excellent technical assistance. Zaira Heredia is the recipient of an FPU contract (FPU15-03644) from the Ministerio de Ciencia, Innovación y Universidades, and she also thanks the University of Malaga for funding the research short-stay at the University of Zurich. This work was supported by AGL2017-83368-C2-I-R grant from the Ministerio de Economía and UMA18-FEDERJA-046 grant from Junta de Andalucía, both partially supported by the European Union (FEDER).

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Author contributions:

ZHP, LE, AV and FMC conceived the study; ZHP, LE, AV and FMC and JAGB designed the experiments; ZHP performed the main experimental work; ZHP realized the data acquisition and data analysis; ZHP and JAGB interpreted the data; GPM assisted in flow-cell chamber experiments; ZHP wrote the manuscript and AV, FMC, JAGB and LE contributed critically to writing the final version of the manuscript. All the authors read and approved the final manuscript.

Conflict of interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials:

The data that support the findings of this study have been included in this manuscript. Original files are available from the corresponding author upon reasonable request. The accession numbers of the sequences used in this study have been included in the Supplementary Material.

Figure legends:

Figure 1. Phylogeny of the *psl*-like genomic cluster into the *P. fluorescens* complex. A) Neighbour-joining tree generated with MEGAX using the combined sequences of the *gyrB* and *rpoD* housekeeping genes. The analysis included 50 strains belonging to the *fluorescens* (24 strains; orange), *gessardii* (5 strains; red), *mandelii* (3 strains; dark blue), *jessenii* (3 strains; light blue), *koreensis* (3 strains; yellow), *corrugata* (3 strains; dark green),

chlororaphis (3 strains; purple) and *protegens* (6 strains; grey) phylogroups. The PcPCL1606 strain is highlighted in bold. The PssUMAF0158 strain (light green) was also included in the analysis. *P. aeruginosa* PAO1 was used as an outgroup (pink). B) Neighbour-joining tree generated with MEGAX using the *psl*-like cluster nucleotide sequence. The analysis included 27 strains belonging to the *fluorescens* (16 strains; orange), *gessardii* (4 strains; red), *mandelii* (1 strain; dark blue), *chlororaphis* (3 strains; purple) and *protegens* (3 strains; grey) phylogroups. The PcPCL1606 strain is highlighted in bold. PssUMAF0158 strain (light green) was also included in the analysis. *P. aeruginosa* PAO1 was used as outgroup (pink).

Figure 2. Early bacterial attachment to abiotic surfaces. The initial attachment (4 h) to abiotic surfaces was assessed using a crystal violet (CV) staining assay. 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 (PcPCL1606 wt), PcPCL1606 Fap-like fibre mutant ($\Delta fapBC$), PcPCL1606 alginate mutant ($\Delta alg8$), PcPCL1606 Psl-like mutant ($\Delta pslE$), PcPCL1606 $\Delta alg8,pslE$ double mutant ($\Delta alg8,pslE$), PcPCL1606 $\Delta alg8,fapBC$ double mutant ($\Delta fapBC,alg8$), PcPCL1606 $\Delta pslE,fapBC$ double mutant ($\Delta pslE,fapBC$), PcPCL1606 $\Delta alg8,pslE,fapBC$ triple mutant ($\Delta alg8,pslE,fapBC$), Fap-like complemented strain ($\Delta fapBC$ +pBBR*fapBC*), alginate complemented strain ($\Delta alg8$ +pBBR*alg8*) and Psl-like complemented strain ($\Delta pslE$ +pBBR*pslEF*) were tested. The complemented strains restored the mutants phenotype to wild-type levels. The dotted line represents the wild-type average. Three technical replicates, and three independent experiments were performed. Statistical analysis was performed using one-way ANOVA with the Bonferroni correction test. Different letters represent statistically significant differences. Error bars show the standard error (SE).

Figure 3. Visualization of flow-cell chamber biofilms of the *P. chlororaphis* PCL1606 wild-type strain and its derived extracellular matrix mutants under CLSM. A) Representative 48 h 3D images of the biofilms formed by the GFP-tagged PcPCL1606 wild-type strain and its derived extracellular matrix mutants are shown. The obtained images were recorded and analysed with the Leica Application Suite (Mannheim, Germany) and the IMARIS software package (Bitplane, Switzerland). Scale bar: 20 μ m. B) Box-and-Whisker plot showing the area in the field of view covered by 48 h biofilms of the GFP-tagged PcPCL1606 wild-type and GFP-tagged extracellular matrix mutants. The cross symbol (x) represents the arithmetic mean. C) Box-and-Whisker plot showing the volume in the field of view occupied by 48 h biofilms of the GFP-tagged PcPCL1606 wild-type and GFP-tagged extracellular matrix mutants. The cross symbol (x) represents the arithmetic mean. The *P.*

823 *chlororaphis* PCL1606 wild-type (PcPCL1606 wt), PcPCL1606 Fap-like fibre mutant
 824 ($\Delta fapBC$), PcPCL1606 alginate mutant ($\Delta alg8$), PcPCL1606 Psl-like mutant ($\Delta pslE$),
 825 PcPCL1606 $\Delta alg8,pslE$ double mutant ($\Delta alg8,pslE$), PcPCL1606 $\Delta alg8,fapBC$ double mutant
 826 ($\Delta fapBC,alg8$), PcPCL1606 $\Delta pslE,fapBC$ double mutant ($\Delta pslE,fapBC$) and PcPCL1606
 827 $\Delta alg8,pslE,fapBC$ triple mutant ($\Delta alg8,pslE,fapBC$) were tested. Three technical replicates,
 828 and a minimum of two independent experiments were performed with each strain. Statistical
 829 analysis was performed using one-way ANOVA with the Bonferroni correction test. Different
 830 letters represent statistically significant differences. Error bars show the distribution of the
 831 numerical data.

832 **Figure 4. *Musa paradisiaca* lectin (BanLec) staining of the *P. chlororaphis* PCL1606**
 833 **extracellular matrix.** Lectin staining of the extracellular matrix of the mCherry-tagged
 834 PcPCL1606 wild-type (red) and its mCherry-tagged Psl-like exopolysaccharide mutant (red)
 835 using FITC-tagged BanLec (green) was performed. The 48h mCherry-tagged PcPCL1606
 836 wild-type (PcPCL1606 wt) and mCherry-tagged PcPCL1606 Psl-like mutant ($\Delta pslE$) biofilms
 837 were tested and observed under CLSM. Three technical replicates, and three independent
 838 experiments were performed. Scale bar: 20 μ m.

839 **Figure 5. Competition in mixed biofilms.** The roles of the Fap-like fibre, alginate and Psl-
 840 like exopolysaccharides in competition during biofilm formation were analysed using the
 841 flow-cell system, and visualization was performed under CLSM. A) Representative 48 h 3D
 842 biofilm images of mixed biofilms including the mCherry-tagged PcPCL1606 wild-type and
 843 each GFP-tagged extracellular matrix mutant. B) Percentage of area occupied by the
 844 PcPCL1606 wild-type (red) and each extracellular matrix mutant (green) after 48 h of
 845 competition. C) Percentage of volume occupied by the PcPCL1606 wild-type (red) and each
 846 extracellular matrix mutant (green) after 48 h of competition. Both area and volume values
 847 were obtained with IMARIS software (Bitplane, Switzerland). The mCherry-tagged *P.*
 848 *chlororaphis* PCL1606 wild-type (PcPCL1606 wt) and GFP-tagged extracellular matrix
 849 PcPCL1606 Fap-like fibrils mutant ($\Delta fapBC$), PcPCL1606 alginate mutant ($\Delta alg8$),
 850 PcPCL1606 Psl-like mutant ($\Delta pslE$), PcPCL1606 $\Delta alg8,pslE$ double mutant ($\Delta alg8,pslE$),
 851 PcPCL1606 $\Delta alg8,fapBC$ double mutant ($\Delta fapBC,alg8$), PcPCL1606 $\Delta pslE,fapBC$ double
 852 mutant ($\Delta pslE,fapBC$) and PcPCL1606 $\Delta alg8,pslE,fapBC$ triple mutant ($\Delta alg8,pslE,fapBC$)
 853 were tested. Three technical replicates, and a minimal of three independent experiments were
 854 performed. —Statistical significance was assessed by two-tailed Mann–Whitney test
 855 (***) $p < 0.001$). Error bars correspond to the standard error (SE). Scale bar: 20 μ m.

Figure 6. Biocontrol of *R. necatrix*-induced white root rot on avocado plants. The PcPCL1606 wild-type strain and *ΔfapBC* and *ΔpslE* extracellular matrix mutants were tested in the *R. necatrix*-avocado test system. The roots of avocado seedlings were inoculated with each strain before being infected with *R. necatrix*. Plants that were not inoculated but were infected were included as negative controls for biocontrol activity. Aerial symptoms were recorded on a scale from 0 to 3 as described in Cazorla *et al.*, 2006. The data collected were used for calculation of the disease index (A) and area under the disease progress curve (B), as described in Cazorla *et al.*, 2006. A) Disease index percentage. The graph shows the time course of the disease index of the avocado plants inoculated and infected with *R. necatrix* (green, red, purple, blue, yellow) and just infected with *R. necatrix* (●black). PcPCL1606 wild-type (■PcPCL1606 wt; green), PcPCL1606 Fap-like mutant strain (◆*ΔfapBC*; purple), PcPCL1606 Psl-like mutant strain (▲*ΔpslE*; red), PcPCL1606 Fap-like mutant complemented strain (●*ΔfapBC* + pBBR*fapBC*; yellow) and PcPCL1606 Psl-like mutant complemented strain (▼*ΔpslE* + pBBR*pslEF*; blue) were tested. B) Area under the disease progress curve (AUDPC) calculated as described in Cazorla *et al.*, 2006. A minimum of seven plants per experiment and two independent experiments were performed. Statistical analysis was performed using one-way ANOVA with the Bonferroni correction test. Different letters represent statistically significant differences. Error bars show the standard error (SE). C) Representative images of bacterial adherence to avocado roots 4 h postinoculation. The PcPCL1606 wild-type (PcPCL1606 wt), PcPCL1606 Fap-like mutant strain (*ΔfapBC*) and PcPCL1606 Psl-like mutant strain (*ΔpslE*) were tested. Three plants per experiment and strain, and three independent experiments were performed. Scale bar: 10 μm.

Role of extracellular matrix components in the formation of biofilms and their contribution to the biocontrol activity of *Pseudomonas chlororaphis* PCL1606

Heredia-Ponce, Zaira¹; Gutiérrez-Barranquero, José Antonio¹; Purtschert-Montenegro, Gabriela²; Eberl, Leo²; de Vicente, Antonio¹; Cazorla, Francisco M.¹

¹Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora” (IHSM-UMA-CSIC) - Departamento de Microbiología, Universidad de Málaga, Bulevar Louis Pasteur, 31 (Campus Universitario de Teatinos), 29071, Málaga, Spain.

²Department of Plant and Microbial Biology. University of Zurich. Zollikerstrasse 107. CH-8008, Zurich, Switzerland.

Corresponding author: Francisco M. Cazorla; email: cazorla@uma.es; Telephone number: [+34 952137587](tel:+34952137587); address: Departamento de Microbiología de la Universidad de Málaga, Bulevar Louis Pasteur, 31 (Campus Universitario de Teatinos), 29071, Málaga (Spain).

Running title: biological role of the EMC of *P. chlororaphis*

Originality-significance statement:

This work is important since it constitutes the first study of the extracellular matrix of the biocontrol agent *P. chlororaphis*. Psl polysaccharide has only been studied in *Pseudomonas aeruginosa* and *Pseudomonas syringae*, in which it plays important roles during biofilm formation. Unprecedented, this work describes the role of a Psl-like exopolysaccharide in the biofilm architecture of a plant-beneficial bacterium. Furthermore, the contribution of a putative Fap-like fibre and Psl-like exopolysaccharide to the biocontrol activity of a strain of *P. chlororaphis* is described for the first time.

Summary:

Pseudomonas chlororaphis PCL1606 (PcPCL1606) displays plant-colonizing features and exhibits antagonistic traits against soil-borne phytopathogenic fungi. Biofilm formation could be relevant for the PcPCL1606 lifestyle, and in this study the role of some putative extracellular matrix components (EMC; Fap-like fibre, alginate and Psl-like polysaccharides) in the biofilm architecture and biocontrol activity of this bacterium were determined. EMC such as the Fap-like fibre and alginate polysaccharide play secondary roles in biofilm formation in PcPCL1606, because they are not fundamental to its biofilm architecture in flow-cell chamber, but synergistically they have shown to favour bacterial competition during biofilm formation. Conversely, studies on Psl-like polysaccharide have revealed that it may

contain mannose, and that it is strongly involved in the PcPCL1606 biofilm architecture and niche competition. Furthermore, the Fap-like fibre and Psl-like exopolysaccharide play roles in early surface attachment and contribute to biocontrol activity against the white root rot disease caused by *Rosellinia necatrix* in avocado plants. These results constitute the first report regarding the study of the extracellular matrix of the PcPCL1606 strain and highlight the importance of a putative Fap-like fibre and Psl-like exopolysaccharide produced by PcPCL1606 in the biofilm formation process and interactions with the host plant root.

Introduction:

Pseudomonas chlororaphis is a common inhabitant of the root environment that belongs to the *Pseudomonas fluorescens* complex (Garrido-Sanz *et al.*, 2017), one of the most diverse bacterial groups within the *Pseudomonas* genus. *P. chlororaphis* typically possesses plant-colonizing activities and exhibits antagonistic traits against soil-borne fungal pathogens (Arrebola *et al.*, 2019). In particular, the *P. chlororaphis* PCL1606 strain (PcPCL1606), isolated from the avocado rhizosphere, has protective features against the soil-borne phytopathogenic fungi *Rosellinia necatrix* and *Fusarium oxysporum*, which produce white root rot disease in avocado and foot and root rot in tomato, respectively (Cazorla *et al.*, 2006; González-Sánchez *et al.*, 2010; Calderón *et al.*, 2015). PcPCL1606 biocontrol activity is mediated by the action of antimicrobial compounds such as proteases and lipases, but mainly production of the compound 2-hexyl 5-propyl resorcinol (HPR), as a direct correlation between the ability to produce this metabolite and a strong inhibition activity against *R. necatrix* and *F. oxysporum* have been observed (Cazorla *et al.*, 2006; Calderón *et al.*, 2013).

The antifungal compound HPR is also involved in the multitrophic interaction avocado root-*R. necatrix*-PcPCL1606 (Calderón *et al.*, 2013, 2014), as well as in biofilm formation and root colonization (Calderón *et al.*, 2014, 2019). Biofilms are accretions of matrix-enclosed microorganisms that adhere to surfaces of biological or non-biological nature. Rhizosphere colonization is favored by the production of an extracellular matrix (Lasík *et al.*, 1989; Matthysse *et al.*, 1998; Lugtenberg *et al.*, 2001; Danhorn & Fuqua *et al.*, 2007; De Weert *et al.*, 2007), but the role that PcPCL1606 extracellular matrix components could play in biofilm formation and the lifestyle of this bacterium remain unknown. The formation of biofilms is a 3-stage process that includes the following steps: 1) attachment to a surface, 2) aggregation of cells, growth and maturation and 3) dispersion (Hall-Stoodley *et al.*, 2004). The first stage consists of adhesion to a surface, and proteinaceous structures such as bacterial adhesins and appendages are fundamental actors in this process. Regarding the protein component of the

65 biofilm matrix of *Pseudomonas*, amyloid-like structures have been gaining importance during
 66 the last decade (Dueholm *et al.*, 2010, 2013; Taglialegna *et al.*, 2016). In particular, Fap
 67 fimbriae could have environmental relevance due to their potential role in plant root
 68 colonization (Dueholm *et al.*, 2013; Rouse *et al.*, 2018). Exopolysaccharides are the main
 69 matrix components necessary for cell aggregation and biofilm maturation. In general,
 70 *Pseudomonas* spp. produces several polysaccharide biofilm matrix molecules, including
 71 alginate, levan, cellulose and Psl polysaccharide (Fuchs *et al.*, 1956; Conti *et al.*, 1994;
 72 Bianciotto *et al.*, 2001; Spiers *et al.*, 2003, 2005; Friedman & Kolter, 2004; Jackson *et al.*,
 73 2004; Matsukawa & Greenberg, 2004). Alginate is composed of β -1,4 linked monomers of L-
 74 glucuronic and D-mannuronic acids (Evans & Linker, 1973), and although it is not
 75 fundamental for the constitution of biofilms in *Pseudomonas aeruginosa* and *Pseudomonas*
 76 *syringae* (Wozniak *et al.*, 2003; Laue *et al.*, 2006; McIntyre-Smith *et al.*, 2010; Heredia-
 77 Ponce *et al.*, 2020), its participation in protection against stressors and biofilm structure have
 78 been proven in these species (Schnider-Keel *et al.*, 2001; Hentzer *et al.*, 2001; Pier *et al.*,
 79 2001; Leid *et al.*, 2005; Heredia-Ponce *et al.*, 2020). Levan polysaccharide, a polymer of β -
 80 2,6 linked fructose, has been mainly studied in *P. syringae*, where the involvement in biofilm
 81 architecture does not seem to be relevant (Laue *et al.*, 2006). Rather, levan accumulation in
 82 cell free areas of biofilms suggests a role as a storage molecule (Laue *et al.*, 2006). Cellulose
 83 is a glucose polymer with β -1,4 glycosidic linkages, which is considered to be an important
 84 biofilm matrix molecule in many environmental pseudomonads (Spiers *et al.*, 2002, 2003,
 85 2005; Mann & Wozniak *et al.*, 2012). Although several biosynthesis and regulation
 86 mechanisms have been described for bacterial cellulose, a common role of this component is
 87 to facilitate the establishment of efficient host-bacteria interactions (Augimeri *et al.*, 2015).
 88 The described Psl polysaccharide, composed of D-mannose, D-glucose and L-rhamnose
 89 (Friedman & Kolter, 2004; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Byrd *et al.*,
 90 2009), has been mainly studied in *P. aeruginosa* (Ma *et al.*, 2009; Zhao *et al.*, 2014; Wang *et al.*,
 91 *et al.*, 2013, 2014; Yang *et al.*, 2018). Recently, its involvement in the biofilm architecture,
 92 virulence and motility have also been described in the plant-associated bacterium *P. syringae*
 93 *pv. syringae* (Heredia-Ponce *et al.*, 2020). Although the presence of the *psl*-like gene cluster
 94 has been reported in some environmentally relevant pseudomonads (Records & Gross, 2010;
 95 Mann & Wozniak *et al.*, 2012), including the biocontrol agent *P. fluorescens* Pf5, the
 96 biological participation of this polysaccharide in beneficial plant-bacteria interactions remains
 97 undetermined.

In this study, we identified a genomic region that putatively encodes a Fap-like amyloid fibre and two genomic regions that putatively encode alginate and Psl-like exopolysaccharides in the PcPCL1606 strain. Since biofilm formation could play a relevant role in the PcPCL1606 lifestyle, research regarding these biofilm components is explored in this work. Thus, the main aim of this work was to study the potential involvement of the Fap-like, alginate and Psl-like-encoding regions in biofilm formation and the lifestyle of PcPCL1606 during its interactions with the host plant root.

Results:

Bioinformatics analysis of the *P. chlororaphis* PCL1606 genome revealed the presence of gene clusters encoding a putative Fap fibre, an alginate and Psl-like exopolysaccharides.

The presence of a Fap-like-, alginate- and Psl-like-encoding clusters has been reported in the PcPCL1606 strain for the first time. An *in-silico* analysis was performed to identify and characterize the genomic regions that might encode these components in PcPCL1606. Using the *P. aeruginosa* PAO1 (PAO1) PA1951-PA1956 genomic region as a template, which encodes the Fap system, the PCL1606_RS15320-PCL1606_RS15345 genomic region of PcPCL1606 was found to show similarity (Supplementary Figure S1A). These similarities were even higher when the *fap* clusters of the *P. fluorescens* UK4 strain (PfUK4), the first strain in which the *fap* operon was described (Dueholm *et al.*, 2010), and PcPCL1606 were compared (Supplementary Figure S1B). The FapC protein constitutes the main protein of the fibre and contains three proteinic repeat motifs (R1-R3; Dueholm *et al.*, 2010). Clustal alignments were performed between each of the three proteinic repeat motifs (R1-R3) present in the FapC protein of PfUK4 and the putative FapC protein of the PcPCL1606 strain (Supplementary Figure S1C). These alignments revealed high conservation patterns among the amino acids present in those regions (Supplementary Figure S1C).

Regarding exopolysaccharide production clusters, the PFLU0300-PFLU0309 genomic region of the *P. fluorescens* SBW25 (SBW25) strain and the Psyrmg_RS20465–Psyrmg_RS20505 genomic region of the phytopathogen *P. syringae* pv. *syringae* UMAF0158 (PssUMAF0158) strain, which correspond to cellulose operon sequences, were not orthologous to any region located in the genome of the PcPCL1606 strain. The PCL1606_RS24735-PCL1606_RS24790 genomic region of PcPCL1606 was orthologous to the PA3540-PA3551 genomic region of PAO1, which encodes proteins of the alginate operon (Supplementary Figure S2). Additionally, the *psl* operon sequence of PAO1 and *psl*-like gene cluster of PssUMAF0158, which correspond to the PA2231-PA2245 and Psyrmg_RS06720-Psyrmg_RS06770 genomic

regions, respectively, allowed us to identify the orthologous PCL1606_RS04710-PCL1606_RS04765 genomic region in PcPCL1606 (Supplementary Figure S3). As previously described in PssUMAF0158 (Heredia-Ponce *et al.*, 2020), the gene that encodes the PslO protein is missing and the PslN-like (PCL1606_RS12970) protein seem to be encoded somewhere else in the chromosome outside the *psl*-like gene cluster (Supplementary Tables S1A-C). The PslM-like (PCL1606_RS04265) protein, which is missing in PssUMAF0158, seems to be encoded somewhere else in the chromosome outside the *psl*-like gene cluster in PcPCL1606 (Supplementary Tables S1A-C). Furthermore, the PAO1 PslL acyltransferase has not been associated with any protein encoded in the PcPCL1606 genome, as it was previously reported in PssUMAF0158 (Heredia-Ponce *et al.*, 2020). However, the PCL1606_RS04715 gene, which is located within the *psl*-like gene cluster, encodes a putative acetyltransferase that might be performing a related function (Supplementary Table S1B). This acetyltransferase shares approximately 60% identity with an acetyltransferase of PssUMAF0158 encoded by an equivalent gene within the *psl*-like gene cluster. Most of the protein domains encoded within the *psl* cluster of PAO1 and *psl*-like gene cluster of PssUMAF0158 are conserved in PcPCL1606 (Supplementary Tables S1A-C).

Phylogenetic analysis revealed that the *psl*-like gene cluster has a random distribution within the *P. fluorescens* complex. To elucidate the phylogeny of the *psl*-like gene cluster within the *P. fluorescens* complex, a total of 50 strains belonging to the different phylogenetic groups included within the *P. fluorescens* complex and isolated from different habitats were selected and used for the analysis (Supplementary Table S2). The recently described *psl*-like gene cluster of PssUMAF0158 was also included in the analysis. The combined nucleotide sequences of the *gyrB* and *rpoD* housekeeping genes clearly supported the phylogenetic distribution in the different phylogroups included in the *P. fluorescens* complex (Garrido-Sanz *et al.*, 2016). The *psl*-like gene cluster was absent in the strains included in Garrido-Sanz *et al.*, 2017 as belonging to the *corrugata*, *jessenii* and *koreensis* phylogroups. Of all the strains included in the *mandelii* phylogroup, only *Pseudomonas* GM21 presented the *psl*-like gene cluster. Except for the *chlororaphis* phylogroup, in which the *psl*-like gene cluster was present in all the strains tested (Supplementary Table S3), the rest of phylogroups contained strains that presented the cluster and strains that did not. The phylogenetic distribution of the strains selected based on the housekeeping genes (Figure 1A) did not correlate with their phylogenetic distribution based on the *psl*-like gene cluster (Figure 1B).

The putative Fap fibre and a Psl-like exopolysaccharide of *P. chlororaphis* PCL1606 are involved in the initial attachment to abiotic surfaces. To investigate the role of the extracellular matrix components under study at the initial stages of PcPCL1606 biofilm formation, short-term adhesion experiments to polystyrene microtiter wells and Crystal violet staining were performed. The results showed that both the Fap-like fibre and the Psl-like exopolysaccharide, but not alginate, were involved in the initial surface attachment (Figure 2). The impaired attachment values were not accumulative for the $\Delta psIE$ and $\Delta fapBC$ mutants, as we observed up to 60% reduction of the attachment levels for the single, double and triple mutants that included the *psIE* gene deletion and up to a 50% reduction for the single and double mutants that included the *fapBC* genes deletion. The complemented strains showed a restoration of the mutant phenotype to wild-type levels.

Production of the Psl-like exopolysaccharide is essential for biofilm formation in *P. chlororaphis* PCL1606. To investigate the roles of the Fap-like fibre, alginate and Psl-like exopolysaccharides in the biofilm architecture of the PcPCL1606 strain, flow-cell chamber experiments were performed, and the biofilm architecture of the wild-type strain was compared to that of its derived extracellular matrix mutants (Figure 3). Confocal laser scanning microscopy (CLSM) and IMARIS software reconstruction allowed us to display representative 3D images of the formed biofilms in the flow-cells. After 48 h, the $\Delta fapBC$ and $\Delta alg8$ single mutants, as well as the $\Delta fapBC, alg8$ double mutant, formed biofilms that were undistinguishable from the wild-type strain (Figure 3A). The biofilms formed by these strains were characterized by a multilayer of gathered cells that covered the flow-cell chamber surface, and the presence of uncolonized space among the aggregating cells were barely noticeable (Figure 3A). In contrast, the biofilms formed by the $\Delta psIE$ mutant consisted of a monolayer of loosely aggregated cells, where the amount of uncolonized space was more perceptible (Figure 3A). The double and triple mutants that included the *psIE* gene deletion showed the same biofilm phenotype as the $\Delta psIE$ single mutant (Figure 3A). The area and volume of the biofilms formed by the $\Delta fapBC$ and $\Delta alg8$ single mutants, as well as the $\Delta fapBC, alg8$ double mutant, were not altered compared to the wild-type strain (Figure 3B and 3C). All the mutants that contained the *psIE* gene deletion showed an impaired biofilm phenotype, which was characterized by a decrease in surface coverage and volume occupied (Figure 3B and 3C).

The Psl-like exopolysaccharide contains mannose and is allocated to the extracellular matrix of *P. chlororaphis* PCL1606. The Psl polysaccharide of the *P. aeruginosa* PAO1

strain is primarily composed of mannose (Ma *et al.*, 2007; Byrd *et al.*, 2009). Thus, to illustrate the presence of Psl-like exopolysaccharide in the extracellular matrix of PcPCL1606 biofilms, lectin staining of 48 h-old biofilms formed by the wild-type strain and its derived Psl-like exopolysaccharide mutant was performed using FITC-labelled *Musa paradisiaca* lectin (BanLec), which binds to mannose residues (Figure 4). The biofilm formed by the PcPCL1606 wild-type strain was able to bind lectin, while the *ΔpslE* mutant strain was not.

The putative Fap fibre, alginate and Psl-like exopolysaccharides provide a competitive advantage during biofilm formation in *P. chlororaphis* PCL1606. To investigate whether the extracellular matrix components under study participate in bacterial competition and niche colonization features displayed by PcPCL1606, competition experiments in flow-cell chambers were performed using mixed biofilms containing the mCherry-tagged wild-type bacterium and the corresponding GFP-tagged extracellular matrix mutant (Figure 5A). The results were analysed as the percentage of colonized space (area and volume) occupied by the wild-type strain and each mutant (Figures 5B and 5C). Competition assays between the *Δalg8* or *ΔfapBC* single mutants and the wild-type strain revealed equivalent fitness in niche competition due to the biofilm formed by the wild-type and each mutant occupied approximately 50% of the area and volume of the biofilm when both were coinoculated (Figure 5B and 5C). However, the *Δalg8,fapBC* double mutant was significantly affected, as within the mixed biofilm, the wild-type strain occupied significantly higher area and volume than the *Δalg8,fapBC* strain. In addition, the *ΔpslE* single mutant, as well as the double and triple mutants containing the *pslE* gene deletion, were severely impaired in niche colonization compared with the wild-type strain (Figure 5A). Thus, *pslE* gene deletion abrogated the ability of the PcPCL1606 strain to compete for biofilm formation with the wild-type equivalent, as the surface coverage and volume occupied by the mutants within the mixed biofilm were markedly reduced (Figure 5B and 5C).

The Fap-like fibre and the Psl-like exopolysaccharide contribute to the biocontrol activity of the *P. chlororaphis* PCL1606 strain. Biocontrol assays against the avocado white root rot disease caused by *R. necatrix* were conducted on the *ΔpslE* and *ΔfapBC* single mutants because the putative Fap fibre and Psl-like exopolysaccharide showed the strongest effects in biofilm-related phenotypes (Figure 2 and 3). Aerial symptoms were evaluated using the previously described disease scale (Cazorla *et al.*, 2006). When no bacteria were applied to the avocado roots, the plants infected with *R. necatrix* reached a disease index value of approximately 76% on day 28 post-inoculation (Figure 6A). However, when the plants were

previously inoculated with the PcPCL1606 wild-type strain, the disease index reached approximately 48% on the same day (Figure 6A). The plants that had been inoculated with the *ΔfapBC* and *ΔpslE* mutant strains prior to *R. necatrix* inoculation reached disease indices that were similar to those observed when the plants were not inoculated and subsequently higher than those observed when the plants were inoculated with the biocontrol wild-type strain (Figure 6A). The area under the disease progress curve (AUDPC) showed that when the plants were inoculated with the PcPCL1606 wild-type strain, the disease severity was reduced by approximately 50% compared with those that were not previously inoculated (Figure 6B). Instead, when the plants were inoculated with the *ΔfapBC* and *ΔpslE* mutants, disease development appeared similar to that observed in plants that were not previously inoculated (Figure 6B). In summary, biocontrol activity was impaired in the *ΔfapBC* and *ΔpslE* mutants, and it was restored to wild-type levels in the *fapBC* and *pslEF* complemented strains (Figure 6B). In addition, CLSM experiments revealed that the *ΔfapBC* and *ΔpslE* mutants showed alterations in their early attachment to avocado roots compared with the wild-type strain (Figure 6C). The wild-type strain showed wider avocado root attachment than the *ΔfapBC* and *ΔpslE* mutants, with both individual and clumps of cells adhered to the avocado root cells. However, the *ΔfapBC* mutant showed less attachment of the root, the pattern of which was characterized by dispersed cell adhesion located among root cells. Finally, the *ΔpslE* mutant also showed less attachment to the roots, exhibiting an irregular pattern.

Discussion:

Biofilm formation plays an advantageous, if not essential, role in plant-bacteria interactions in many environmentally relevant *Pseudomonas* (Morris & Monier, 2003; Danhorn & Fuqua, 2007). Plant-associated bacteria can interact with the aerial parts, the rhizosphere or even the vascular system of the plant host using a great diversity of extracellular polymeric substances, including exopolysaccharides, proteins and DNA (Ramey *et al.*, 2004). Even though some *P. chlororaphis* strains have been reported as biofilm formers (Maddula *et al.* 2006; Poritsanos *et al.* 2006; Kim *et al.* 2014; Calderón *et al.*, 2019), little attention has been paid to the study of their extracellular matrix components. The *P. chlororaphis* PCL1606 strain (PcPCL1606) is a biofilm-forming bacterium with root-colonization and biocontrol activities (Cazorla *et al.*, 2006; Calderón *et al.* 2019; Arrebola *et al.*, 2019). The PcPCL1606 extracellular matrix could play a relevant role in these processes, and thereby, based on its lifestyle, the involvement of some putative extracellular matrix components in biofilm formation ability and biocontrol activity of this strain were explored in this work.

262 In this study, three genomic clusters were identified in the PcPCL1606 strain, which
 263 putatively encode a Fap amyloid-like fibre and two exopolysaccharides, alginate and Psl
 264 polysaccharide. These genomic regions might be involved in biofilm formation, as their
 265 relevance in other *Pseudomonas* has been previously revealed (Bianciotto *et al.*, 2001;
 266 Friedman & Kolter, 2004; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Franklin *et al.*,
 267 2011; Dueholm *et al.*, 2013; Marshall *et al.*, 2019; Heredia-Ponce *et al.*, 2020). The
 268 genomic regions encoding the Fap fibre and alginate exopolysaccharide have been identified
 269 in several species of *Pseudomonas* (Conti *et al.*, 1994; Stapper *et al.*, 2004; Laue *et al.*, 2006;
 270 Rouse *et al.*, 2018; Marshall *et al.*, 2019). However, the genomic regions encoding a Psl-like
 271 exopolysaccharide have only been identified in *P. aeruginosa*, some strains of *P. syringae*, *P.*
 272 *mendocina* ymp and *P. fluorescens* Pf5 (Mann & Wozniak, 2012; Heredia-Ponce *et al.*, 2020).
 273 Regardless, the biological role of Psl exopolysaccharide in beneficial plant-bacteria
 274 interactions is unknown to date. The results obtained in the phylogenetic analysis (Figure 1)
 275 suggest that the distribution of the *psl*-like gene cluster within *P. fluorescens* complex is
 276 promiscuous. Additionally, the presence of the *psl*-like gene cluster in isolates of different
 277 sources (Supplementary Table S2) further suggests that the ability to produce this
 278 exopolysaccharide may not be habitat-dependent, but a more general feature of biofilm
 279 formation, and that this gene cluster could have been acquired by horizontal gene transfer.
 280 The *psl*-like cluster is widely distributed within the *chlororaphis* phylogroup (Supplementary
 281 Table S3), which may explain why relevant roles in biofilm formation and related aspects
 282 have been observed in the PcPCL1606 strain in this study.

283 The initial stage in the biofilm formation process consists of attachment to a surface (Hall-
 284 Stoodley *et al.*, 2004). Proteinaceous structures, such as bacterial adhesins and appendages,
 285 play fundamental roles in this process (Berne *et al.*, 2015). Although exopolysaccharides were
 286 thought to be mainly required for later architectural development of the biofilms (Sutherland,
 287 1999), implications in the initial attachment to the surfaces of roots have also been reported in
 288 several species (Wheatley *et al.*, 2018). In agreement with previous studies (Colvin *et al.*,
 289 2012; Berne *et al.*, 2015), the Fap-like fibre and Psl-like exopolysaccharide of the PcPCL1606
 290 strain were found to be involved in the initial attachment to abiotic surfaces (Figure 2). The
 291 finding that alginate did not play an important role in PcPCL1606 during initial surface
 292 attachment (Figure 2) was also consistent with other studies performed in *P. aeruginosa*, in
 293 which this polysaccharide was not truly required for the early steps of biofilm formation
 294 (Wozniak *et al.*, 2003; McIntyre-Smith *et al.*, 2010).

Regarding later stages of biofilm development, the Psl-like exopolysaccharide was proven to be an essential component of the biofilm architecture of the PcPCL1606 strain (Figure 3). This result was in line with previous studies, in which the Psl polysaccharide was reported to be important for adhesion and maintaining the biofilm structure post-attachment (Friedman & Kolter, 2004; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Ma *et al.*, 2006; Heredia-Ponce *et al.* 2020). After 48 h of incubation, the biofilm phenotype of the PcPCL1606 Δ pslE strain resembled that of the PAO1 Psl-like mutant (Jackson *et al.*, 2004; Ghafoor *et al.*, 2011), consisting of a monolayer of cells (Figure 3A and 3C), which suggests that the Psl-like exopolysaccharide could be decisive for the constitution of the three-dimensional architecture of the PcPCL1606 biofilm. In *P. aeruginosa* PAO1, the most recent study determined that Psl polysaccharide is composed of a pentasaccharide subunit containing D-mannose, D-glucose and L-rhamnose, at approximate ratios of 3:1:1 (Byrd *et al.*, 2009). However, this result conflicts with previous reports, in which the presence of galactose in the Psl structure was also reported (Ma *et al.*, 2007). In any case, mannose is described as the main monosaccharide of the polymer. BanLec staining of the Psl-like exopolysaccharide of the PcPCL1606 strain suggested that it also contained mannose (Figure 4), which is in agreement with prior studies on *P. aeruginosa* and suggests that these polysaccharides might actually be similar in composition. The alginate mutants of *P. aeruginosa*, *P. syringae* and *P. fluorescens* retained the ability to form biofilms compared to their respective wild-types, but their biofilm architectures were different (Wozniak *et al.*, 2003; Stapper *et al.*, 2004; Laue *et al.*, 2006; Ghafoor *et al.*, 2011; Noirot-Gros *et al.*, 2019; Heredia-Ponce *et al.*, 2020). However, no impairment in biofilm architecture was observed in the Δ alg8 mutant strain of PcPCL1606 (Figure 3). The presence of the *fap* operon has been reported in several *Pseudomonas* species (Mann & Wozniak *et al.*, 2012; Dueholm *et al.*, 2013), but its role in biofilm formation has been considered as secondary, because the PAO1 Fap mutant formed biofilms to the same extent as the wild-type strain (Dueholm *et al.*, 2013). In line with the published data, and despite the differences observed in early adhesion in the Δ fapBC mutant (Figure 2 and 6C), similar surface coverage and volumes values of the biofilms formed by the PcPCL1606 wild-type strain and Δ fapBC mutant have been observed (Figure 3), leading to a similar conclusion. Thus, similar to *P. aeruginosa* (Dueholm *et al.*, 2013), the role of the PcPCL1606 Fap-like fibre seems to be more directed towards initial attachment of cells to surfaces, as the early adhesion deficiencies of the Δ fapBC mutant (4 h) were not observed at later time periods (6 and 8 h; Supplementary Figure S4) and the developed biofilm architecture of the mutant was indistinguishable from that of the wild-type (Figure 3).

329 The ability to produce Psl-like polysaccharide provided a selective advantage to synthesizing
 330 bacteria in competition for biofilm formation (Figure 5). This result had been previously
 331 observed in *P. aeruginosa*, in which the Δpsl mutant of PAO1 strain could not be rescued by
 332 the parental strain when both were co-cultured (Jackson *et al.*, 2004; Irie *et al.*, 2016).
 333 Interestingly, these competition experiments suggest that Psl is acting as a private good in
 334 PcPCL1606, as it has been previously reported in *P. aeruginosa* (Irie *et al.*, 2016). Instead, the
 335 Fap-like fibre and the alginate exopolysaccharide were shown to play secondary roles in
 336 biofilm formation of the PcPCL1606 strain, as the biofilms formed by the mutant strains
 337 showed surface coverage and volume values that were undistinguishable from those of the
 338 wild-type (Figure 3), but together they contributed synergistically to biofilm architecture by
 339 providing colonization advantages during biofilm formation (Figure 5). This result correlates
 340 with the impaired phenotype in early attachment observed in the $\Delta fapBC, alg8$ double mutant
 341 (Figure 2). Cooperation between different extracellular matrix components is not uncommon,
 342 as some of them have been described to only function synergistically during biofilm
 343 formation (Saldaña *et al.*, 2009; Ostrowski *et al.*, 2011; Fong *et al.*, 2015).

344 Colonization of the roots is one of the first steps in the establishment of beneficial plant-
 345 bacteria interactions (De Weert & Bloemberg, 2007). Since PcPCL1606 is a biocontrol agent
 346 of plant-fungal diseases, it was essential to ascertain the contribution of the Fap-like fibrils
 347 and the Psl-like exopolysaccharide, which showed the strongest effects in biofilm related
 348 phenotypes, in disease control. Both biofilm components were found to be relevant in white
 349 root rot disease suppression (Figures 6A and 6B). The putative roles of Psl polysaccharide in
 350 the surface colonization of *P. aeruginosa* (Zhao *et al.*, 2014) and *P. syringae* (Heredia-Ponce
 351 *et al.*, 2020), as well as the presence of a *psl*-like gene cluster in some pseudomonads that
 352 usually colonize the soil and roots of plants (Supplementary Table S2), revealed the potential
 353 involvement of the PcPCL1606 Psl-like exopolysaccharide in the interaction with the plant
 354 root. There is evidence suggesting that the Fap system could also play a role in the interaction
 355 with the plant root (Dueholm *et al.*, 2013; Rouse *et al.*, 2018). The obtained results revealed a
 356 clear influence of the Fap-like fibre and the Psl-like exopolysaccharide in PcPCL1606
 357 biocontrol ability (Figures 6A and 6B). Thus, the impairment in both initial abiotic and biotic
 358 surface attachment observed in the $\Delta fapBC$ and $\Delta pslE$ mutants (Figure 2 and 6C), as well as
 359 the contribution of the Psl-like exopolysaccharide to the PcPCL1606 overall biofilm
 360 architecture (Figures 3 and 5), could all indicate that inefficient interaction with the plant root
 361 by the mutants might actually lead to an impairment of disease suppression, probably by
 362 allowing a better access of the fungus to the plant root or by providing an ineffective delivery

system of antifungal metabolites, as previously suggested (Chin-A-Woeng *et al.*, 2007). Thus, effective colonization of the rhizosphere appears as a prerequisite for the efficient disease control of PcPCL1606, in agreement with former studies performed in biocontrol agents (Bloemberg & Lugtenberg, 2001; Lugtenberg & Kamilova, 2009).

In conclusion, our work constitutes the first report regarding the presence of a *psl*-like gene cluster within the *P. chlororaphis* group, its role in biofilm formation and influence on the biocontrol lifestyle in the plant-beneficial bacterium PcPCL1606. Furthermore, the Fap-type system and alginate exopolysaccharide of PcPCL1606, although not fundamental for biofilm formation, seem to cooperatively contribute to biofilm formation in this bacterium by proving relevance during competitive colonization in biofilm formation experiments. Additionally, this is the first time that the role in early surface interactions and the biocontrol lifestyle has been demonstrated for a Fap-like fibre, in a manner similar to that of the Psl-like exopolysaccharide. These results contribute to a better understanding of the biofilm matrix of the PcPCL1606 biocontrol strain and its involvement in the multitrophic interactions that take place during the biological control of soil-borne plant pathogens.

Experimental procedures:

• Bacterial strains and growth conditions:

The bacterial strains and plasmids used in this study are listed in Supplementary Table S4. For standard maintenance, PcPCL1606 (Cazorla *et al.*, 2006) and the extracellular matrix mutants were grown in tryptone-peptone-glycerol medium (TPG; Calderón *et al.*, 2014a) at 25°C. *Escherichia coli* was used as a host for the mutation and complementation plasmids and was routinely grown in lysogenic broth (LB) at 37°C. The antibiotics used for the selection of *P. chlororaphis* derivatives were kanamycin 50 mg/L (Km₅₀) and gentamycin 50 mg/L (Gm₅₀). The antibiotics used for the selection of *E. coli* derivatives were kanamycin 50 mg/L (Km₅₀), ampicillin 100 mg/L (Ap₁₀₀) and gentamycin 10 mg/L (Gm₁₀). TPG medium was used for the *in vitro* experiments. Pseudomonas Isolation Agar (PIA; Difco) was used for PcPCL1606 fluorescent tagging by triparental conjugation. Flow-cell chamber experiments were performed using ABC minimal medium (Clark & Maaløe, 1967).

• Bioinformatics analysis:

Nucleotide and protein sequence searches were performed with the Pseudomonas Genome Database (<https://www.pseudomonas.com/>) and the National Center for Biotechnology

Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>). Putative protein domain searches were carried out using Protein Family Software (PFAM) (<https://pfam.xfam.org/>).

• **Construction of bacterial mutants and tagging:**

P. chlororaphis PCL1606 (PcPCL1606) knockout extracellular matrix mutants were generated according to the protocol described in Zumaquero *et al.*, 2010. Specific primers are listed in Supplementary Table S5. The transformants were selected on TPG medium containing kanamycin. To assess whether each transconjugant integrated the plasmid into the chromosome or engaged in allelic exchange, PCR amplifications of a fragment within the selected gene and southern blot analysis using a fragment within the gene and the Km cassette as probes were performed. To create the doubles and triple mutants, the kanamycin cassette was removed using a modified pFPL2 plasmid, in which the gentamycin cassette present in the pBBR1MCS5 plasmid was amplified and introduced into the pFLP2 plasmid using primers containing the BamHI and XbaI restriction sequences (Supplementary Table S5). The complementation plasmids were constructed using specific primers that included the amplification of the fifty base pairs upstream of the selected gene, to include their own ribosome binding site. For *pslE* gene complementation, both the *pslE* and *pslF* genes were amplified. The resulting amplicons were then cloned into the pBBR1MCS5 plasmid and fully sequenced to discard any possible mutations. Fluorescent tagging was performed using the triparental conjugation protocol described in Lambertsen *et al.*, 2004. Briefly, the *E. coli* S17-1 donor, *E. coli* S17-1 helper and recipient strains were grown overnight at an appropriate temperature (37°C for *E. coli* and 25°C for *Pseudomonas*). Then, the bacterial suspensions were washed (1x) with 0.85% NaCl solution. Subsequently, 200 µl of each donor and helper, and 100 µl of the recipient strain, were mixed, plated in LB-agar and incubated overnight at 25°C. The conjugation spots were subsequently resuspended in 0.85% NaCl solution, serially diluted 10-fold and spread on PIA plates to avoid *E. coli* growth. The plates were incubated 48 h at 25°C for single fluorescent PcPCL1606 colonies to appear.

• **Phylogenetic analysis:**

The phylogenetic analyses were performed using combined sequences of the *gyrB* and *rpoD* housekeeping genes, along with the complete nucleotide sequence of the *psl*-like gene clusters. The *psl*-like gene clusters were identified performing megablast and discontinuous megablast searches in BLASTN with the PcPCL1606 *psl*-like gene cluster as a model (*pslABCDEFGHIJKL*-like genes), as well as the genomes of fifty strains belonging to the different phylogenetic groups included in the *P. fluorescens* complex (Supplementary Table

S2). Sequence alignment was performed using ClustalW (Larkin *et al.*, 2007). Phylogenetic trees were constructed using MEGAX software with Jukes Cantor's algorithm and the maximum likelihood (ML) statistical method. The confidence level for the branching points was determined using 1,000 bootstrap replicates. *P. aeruginosa* PAO1 was used as an outgroup.

• **Bacterial attachment assay:**

The attachment assays were conducted as previously described (McCarthy *et al.*, 2014; Gutiérrez-Barranquero *et al.*, 2015), with slight modifications. Five hundred microliters of the PcPCL1606 wild-type strain and its derivative extracellular matrix mutant at an OD of 0.25 a.u. at 600 nm (approx. 10^8 CFU/ml) were inoculated in 24-well microtiter plates. To ensure that there were no differences in bacterial concentration at the time of inoculation, cell counts of the bacterial suspensions were performed. After 4, 6 or 8 h of incubation at 25°C, cultures were removed and washed with distilled water. Five hundred microliters of 0.1% crystal violet (Panreac) as added to each well, followed by an incubation period of 15 min. Then, the crystal violet was removed, and the wells were vigorously washed with tap water. The plates were subsequently dried at 37°C for 2 h. Finally, 500 µl of 96% EtOH were added to each well and incubated under agitation at room temperature for 15 min. One hundred microliters was then collected from each well and added to a clean 96-well plate for absorbance measurements at 595 nm. Finally, the fold change data were obtained by normalizing the absorbance values relative to the wild-type average of each independent experiment.

• **Flow-cell assays and biofilm visualization:**

To assess differences regarding biofilm formation between the PcPCL1606 wild-type and its knockout extracellular matrix mutants, flow-cell chambers were prepared and assembled as previously described (Christensen *et al.*, 1999). Fresh GFP-tagged wild-type and GFP-tagged extracellular matrix mutants were inoculated as single colonies into 5 ml of TPG tubes and incubated overnight at 200 rpm and 25°C. Then, the cultures were adjusted to a low cell density with 0.85% NaCl solution (OD of 0.01 a.u at 600 nm, which corresponds to 10^6 CFU/ml), and 300 µl was injected into each channel with a small syringe. After inoculation, the flow remained stopped for 1 h to allow attachment of the cells to the channels. The flow-cells were supplied with ABC minimal medium (Clark & Maaløe, 1967). Medium flow was maintained at a constant rate of 1.3 ml/min by a Watson-Marlow 205S peristaltic pump and the incubation temperature was 25°C. Microscopic inspection and image acquisition were performed using a confocal laser scanning microscope (CLSM; DM5500Q, Leica) equipped

with a 40/1.3 or a 63/1.4 oil objective. Captured images obtained with the Leica Application Suite (Mannheim, Germany) were used to quantify biofilms area and volume values using the IMARIS software package (Bitplane, Switzerland).

- **Lectin staining of Psl-like components:**

The putative presence of a Psl-like polysaccharide within the PcPCL1606 extracellular matrix was assayed using the mCherry-tagged wild-type and the mCherry-tagged *ΔpslE* mutant strain, as well as the FITC-tagged *Musa paradisiaca* lectin (BanLec; Glycomatrix), which binds to mannose residues (Singh *et al.*, 2014). First, the biofilms were grown in flow-cell chambers as mentioned above. BanLec was used at a final concentration of 100 µg/ml, as previously described for lectins (Neu *et al.*, 1999). Forty-eight hours postinoculation, the flow was stopped and 100 µl of a 100 µg/ml lectin solution was gently injected into the chamber, to avoid complete wash out of the biofilms. After 20 min of staining, the flow was restarted to allow the clearance of the unbound protein for 20 min before microscopic observation. Subsequently, the images were recorded and analysed with the Leica Application Suite (Mannheim, Germany) and IMARIS software package (Bitplane, Switzerland). To detect the cells, the 532-nm laser was used for mCherry fluorophore excitation, and emission was monitored at 570-700 nm. The FITC-tagged BanLec was excited with the 488-nm laser, and emission was monitored at 450-500 nm.

- **Competition experiments during biofilm formation:**

The competition experiments during biofilm formation were performed in flow cells using ABC minimal medium (Clark & Maaloe, 1967). The flow-cell system was prepared and assembled as previously described (Christensen *et al.*, 1999). The channels were inoculated with a 1:1 mixture of differentially mCherry-tagged wild-type and GFP-tagged extracellular matrix mutants, previously grown at a low cell density ($OD_{600nm} = 0.01$ a.u., which corresponds to 10^6 CFU/ml). Images were recorded and analysed with the Leica Application Suite (Mannheim, Germany) and IMARIS software package (Bitplane, Switzerland). The 532-nm laser was used for mCherry fluorophore excitation, and emission was monitored at 570-700 nm. The 488-nm laser was used for GFP fluorophore excitation, and emission was monitored at 450-500 nm.

- **Biocontrol assays against avocado white root rot:**

The biocontrol assays against avocado white root rot disease were performed as previously described (Cazorla *et al.*, 2006). The roots of 6-month-old avocado plants obtained from

Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain) were disinfected with 1% v/v household bleach for 20 min, washed with distilled water and inoculated as previously described (Calderón *et al.*, 2013). For the inoculation process, the roots of the avocado plants were immersed either in a suspension of the bacterial isolate (10^8 CFU/ml) or in sterile TPG medium (controls) for 20 min. Excess bacteria were removed from the roots, and the seedlings were transplanted into pots containing potting soil (Jongkind Grond B.V., Aalsmeer, The Netherlands) and subsequently infected with *Rosellinia necatrix* using eight colonized wheat grains per pot (Freeman *et al.*, 1986). The plants that were not inoculated but were infected served as negative controls for biocontrol activity. Furthermore, the plants that were neither inoculated nor infected served as environmental controls. Two independent experiments, each with at least 7 avocado seedlings per strain, were performed. Aerial symptoms were recorded on a scale from 0 to 3 (from 0 – not affected – to 3 – dead plant). The disease index was calculated using the previously described formula (Cazorla *et al.*, 2006), and to minimize the variability inherent to the experiments with plants, all values were normalized to the wild-type average for each independent experiment.

• **Bacterial adhesion to avocado roots:**

The avocado roots were disinfected in 1% v/v household bleach for 20 min, washed in distilled water and immersed in GFP-tagged bacterial cultures at a final concentration of 10^8 CFU/ml for 20 min, as previously described (Calderón *et al.*, 2013). Then, each root was washed again by immersion in distilled water to remove the free and weakly attached bacteria and transplanted into pots filled with vermiculite. After 4 h of incubation, the plants were removed from the pots, and superficial root samples were randomly collected with a scalpel and placed on glass slides for examination. The fluorescently-labelled bacteria attached to the roots were visualized by confocal laser scanning microscopy (CLSM) using a Zeiss LSM 880 microscope. Three independent experiments, each with three avocado seedlings per strain, were performed.

Acknowledgments:

The authors would like to thank Dr. Aurélien Bailly (University of Zurich) for guidance and assistance with the confocal microscopy, Sandra Tienda (University of Malaga) for the help and assistance with the experiments with plants and Irene Linares (University of Malaga) for her excellent technical assistance. Zaira Heredia is the recipient of an FPU contract (FPU15-03644) from the Ministerio de Ciencia, Innovación y Universidades, and she also thanks the

University of Malaga for funding the research short-stay at the University of Zurich. This work was supported by AGL2017-83368-C2-I-R grant from the Ministerio de Economía and UMA18-FEDERJA-046 grant from Junta de Andalucía, both partially supported by the European Union (FEDER).

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Author contributions:

ZHP, LE, AV and FMC conceived the study; ZHP, LE, AV and FMC and JAGB designed the experiments; ZHP performed the main experimental work; ZHP realized the data acquisition and data analysis; ZHP and JAGB interpreted the data; GPM assisted in flow-cell chamber experiments; ZHP wrote the manuscript and AV, FMC, JAGB and LE contributed critically to writing the final version of the manuscript. All the authors read and approved the final manuscript.

Conflict of interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials:

The data that support the findings of this study have been included in this manuscript. Original files are available from the corresponding author upon reasonable request. The accession numbers of the sequences used in this study have been included in the Supplementary Material.

Figure legends:

Figure 1. Phylogeny of the *psl*-like genomic cluster into the *P. fluorescens* complex. A) Neighbour-joining tree generated with MEGAX using the combined sequences of the *gyrB* and *rpoD* housekeeping genes. The analysis included 50 strains belonging to the *fluorescens* (24 strains; orange), *gessardii* (5 strains; red), *mandelii* (3 strains; dark blue), *jessenii* (3 strains; light blue), *koreensis* (3 strains; yellow), *corrugata* (3 strains; dark green),

chlororaphis (3 strains; purple) and *protegens* (6 strains; grey) phylogroups. The PcPCL1606 strain is highlighted in bold. The PssUMAF0158 strain (light green) was also included in the analysis. *P. aeruginosa* PAO1 was used as an outgroup (pink). B) Neighbour-joining tree generated with MEGAX using the *psl*-like cluster nucleotide sequence. The analysis included 27 strains belonging to the *fluorescens* (16 strains; orange), *gessardii* (4 strains; red), *mandelii* (1 strain; dark blue), *chlororaphis* (3 strains; purple) and *protegens* (3 strains; grey) phylogroups. The PcPCL1606 strain is highlighted in bold. PssUMAF0158 strain (light green) was also included in the analysis. *P. aeruginosa* PAO1 was used as outgroup (pink).

Figure 2. Early bacterial attachment to abiotic surfaces. The initial attachment (4 h) to abiotic surfaces was assessed using a crystal violet (CV) staining assay. 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 (PcPCL1606 wt), PcPCL1606 Fap-like fibre mutant ($\Delta fapBC$), PcPCL1606 alginate mutant ($\Delta alg8$), PcPCL1606 Psl-like mutant ($\Delta pslE$), PcPCL1606 $\Delta alg8,pslE$ double mutant ($\Delta alg8,pslE$), PcPCL1606 $\Delta alg8,fapBC$ double mutant ($\Delta fapBC,alg8$), PcPCL1606 $\Delta pslE,fapBC$ double mutant ($\Delta pslE,fapBC$), PcPCL1606 $\Delta alg8,pslE,fapBC$ triple mutant ($\Delta alg8,pslE,fapBC$), Fap-like complemented strain ($\Delta fapBC+pBBRfapBC$), alginate complemented strain ($\Delta alg8+pBBRalg8$) and Psl-like complemented strain ($\Delta pslE+pBBRpslEF$) were tested. The complemented strains restored the mutants phenotype to wild-type levels. The dotted line represents the wild-type average. Three technical replicates, and three independent experiments were performed. Statistical analysis was performed using one-way ANOVA with the Bonferroni correction test. Different letters represent statistically significant differences. Error bars show the standard error (SE).

Figure 3. Visualization of flow-cell chamber biofilms of the *P. chlororaphis* PCL1606 wild-type strain and its derived extracellular matrix mutants under CLSM. A) Representative 48 h 3D images of the biofilms formed by the GFP-tagged PcPCL1606 wild-type strain and its derived extracellular matrix mutants are shown. The obtained images were recorded and analysed with the Leica Application Suite (Mannheim, Germany) and the IMARIS software package (Bitplane, Switzerland). Scale bar: 20 μ m. B) Box-and-Whisker plot showing the area in the field of view covered by 48 h biofilms of the GFP-tagged PcPCL1606 wild-type and GFP-tagged extracellular matrix mutants. The cross symbol (x) represents the arithmetic mean. C) Box-and-Whisker plot showing the volume in the field of view occupied by 48 h biofilms of the GFP-tagged PcPCL1606 wild-type and GFP-tagged extracellular matrix mutants. The cross symbol (x) represents the arithmetic mean. The *P.*

820 *chlororaphis* PCL1606 wild-type (PcPCL1606 wt), PcPCL1606 Fap-like fibre mutant
 821 ($\Delta fapBC$), PcPCL1606 alginate mutant ($\Delta alg8$), PcPCL1606 Psl-like mutant ($\Delta pslE$),
 822 PcPCL1606 $\Delta alg8,pslE$ double mutant ($\Delta alg8,pslE$), PcPCL1606 $\Delta alg8,fapBC$ double mutant
 823 ($\Delta fapBC,alg8$), PcPCL1606 $\Delta pslE,fapBC$ double mutant ($\Delta pslE,fapBC$) and PcPCL1606
 824 $\Delta alg8,pslE,fapBC$ triple mutant ($\Delta alg8,pslE,fapBC$) were tested. Three technical replicates,
 825 and a minimum of two independent experiments were performed with each strain. Statistical
 826 analysis was performed using one-way ANOVA with the Bonferroni correction test. Different
 827 letters represent statistically significant differences. Error bars show the distribution of the
 828 numerical data.

829 **Figure 4. *Musa paradisiaca* lectin (BanLec) staining of the *P. chlororaphis* PCL1606**
 830 **extracellular matrix.** Lectin staining of the extracellular matrix of the mCherry-tagged
 831 PcPCL1606 wild-type (red) and its mCherry-tagged Psl-like exopolysaccharide mutant (red)
 832 using FITC-tagged BanLec (green) was performed. The 48h mCherry-tagged PcPCL1606
 833 wild-type (PcPCL1606 wt) and mCherry-tagged PcPCL1606 Psl-like mutant ($\Delta pslE$) biofilms
 834 were tested and observed under CLSM. Three technical replicates, and three independent
 835 experiments were performed. Scale bar: 20 μ m.

836 **Figure 5. Competition in mixed biofilms.** The roles of the Fap-like fibre, alginate and Psl-
 837 like exopolysaccharides in competition during biofilm formation were analysed using the
 838 flow-cell system, and visualization was performed under CLSM. A) Representative 48 h 3D
 839 biofilm images of mixed biofilms including the mCherry-tagged PcPCL1606 wild-type and
 840 each GFP-tagged extracellular matrix mutant. B) Percentage of area occupied by the
 841 PcPCL1606 wild-type (red) and each extracellular matrix mutant (green) after 48 h of
 842 competition. C) Percentage of volume occupied by the PcPCL1606 wild-type (red) and each
 843 extracellular matrix mutant (green) after 48 h of competition. Both area and volume values
 844 were obtained with IMARIS software (Bitplane, Switzerland). The mCherry-tagged *P.*
 845 *chlororaphis* PCL1606 wild-type (PcPCL1606 wt) and GFP-tagged extracellular matrix
 846 PcPCL1606 Fap-like fibrils mutant ($\Delta fapBC$), PcPCL1606 alginate mutant ($\Delta alg8$),
 847 PcPCL1606 Psl-like mutant ($\Delta pslE$), PcPCL1606 $\Delta alg8,pslE$ double mutant ($\Delta alg8,pslE$),
 848 PcPCL1606 $\Delta alg8,fapBC$ double mutant ($\Delta fapBC,alg8$), PcPCL1606 $\Delta pslE,fapBC$ double
 849 mutant ($\Delta pslE,fapBC$) and PcPCL1606 $\Delta alg8,pslE,fapBC$ triple mutant ($\Delta alg8,pslE,fapBC$)
 850 were tested. Three technical replicates, and a minimal of three independent experiments were
 851 performed. Statistical significance was assessed by two-tailed Mann–Whitney test
 852 (***) $p < 0.001$). Error bars correspond to the standard error (SE). Scale bar: 20 μ m.

Figure 6. Biocontrol of *R. necatrix*-induced white root rot on avocado plants. The PcPCL1606 wild-type strain and *ΔfapBC* and *ΔpslE* extracellular matrix mutants were tested in the *R. necatrix*-avocado test system. The roots of avocado seedlings were inoculated with each strain before being infected with *R. necatrix*. Plants that were not inoculated but were infected were included as negative controls for biocontrol activity. Aerial symptoms were recorded on a scale from 0 to 3 as described in Cazorla *et al.*, 2006. The data collected were used for calculation of the disease index (A) and area under the disease progress curve (B), as described in Cazorla *et al.*, 2006. A) Disease index percentage. The graph shows the time course of the disease index of the avocado plants inoculated and infected with *R. necatrix* (green, red, purple, blue, yellow) and just infected with *R. necatrix* (●black). PcPCL1606 wild-type (■PcPCL1606 wt; green), PcPCL1606 Fap-like mutant strain (◆*ΔfapBC*; purple), PcPCL1606 Psl-like mutant strain (▲*ΔpslE*; red), PcPCL1606 Fap-like mutant complemented strain (●*ΔfapBC* + pBBR*fapBC*; yellow) and PcPCL1606 Psl-like mutant complemented strain (▼*ΔpslE* + pBBR*pslEF*; blue) were tested. B) Area under the disease progress curve (AUDPC) calculated as described in Cazorla *et al.*, 2006. A minimum of seven plants per experiment and two independent experiments were performed. Statistical analysis was performed using one-way ANOVA with the Bonferroni correction test. Different letters represent statistically significant differences. Error bars show the standard error (SE). C) Representative images of bacterial adherence to avocado roots 4 h postinoculation. The PcPCL1606 wild-type (PcPCL1606 wt), PcPCL1606 Fap-like mutant strain (*ΔfapBC*) and PcPCL1606 Psl-like mutant strain (*ΔpslE*) were tested. Three plants per experiment and strain, and three independent experiments were performed. Scale bar: 10 μm.

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180x125mm (600 x 600 DPI)

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