

Characterisation of the biosurfactants from phyllosphere colonising *Pseudomonads* and their effect on plant colonisation and diesel degradation

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Abstract

Biosurfactant production is a common trait in leaf surface colonising bacteria that has been associated with increased survival and movement on leaves. At the same time the ability to degrade aliphatics is common in biosurfactant-producing leaf colonisers. *Pseudomonads* are common leaf colonisers and have been recognised for their ability to produce biosurfactants and degrade aliphatic compounds. In this study, we have investigated the role of biosurfactants in four non-plant pathogenic *Pseudomonas* strains by performing a series of experiments to characterise the surfactant properties, and their role during leaf colonisation and diesel degradation. The produced biosurfactants were identified using mass-spectrometry. Two strains produced viscosin-like biosurfactants and the other two produced Massetolide A-like biosurfactants which aligned with the phylogenetic relatedness between the strains. To further investigate the role of surfactant production, random Tn5 transposon mutagenesis was performed to generate knockout mutants. The knockout mutants were compared to their respective wildtypes in their ability to colonise gnotobiotic *Arabidopsis thaliana* and to degrade diesel. It was not possible to detect negative effects during plant colonisation in direct competition or individual colonisation experiments. When grown on diesel, knockout mutants grew significantly slower compared to their respective wildtypes. By adding isolated wildtype biosurfactants it was possible to complement the growth of the knockout mutants.

41 Importance

42 Many leaf colonising bacteria produce surfactants and are able to degrade aliphatic
43 compounds, however, if surfactant production provides a competitive advantage during leaf
44 colonisation is unclear. Furthermore, it is unclear if leaf colonisers take advantage of the
45 aliphatic compounds that constitute the leaf cuticle and cuticular waxes. Here we test the
46 effect of surfactant production on leaf colonisation and demonstrate that the lack of
47 surfactant production decreases the ability to degrade aliphatic compounds. This indicates
48 that leaf surface dwelling, surfactant producing bacteria contribute to degradation of
49 environmental hydrocarbons and may be able to utilise leaf surface waxes. This has
50 implications for plant-microbe interactions and future studies.

51

52 Introduction

53 The leaf cuticle is a hydrophobic barrier which consist of cutin, a polymer of very long chain
54 aliphatics, interspersed and overlaid by very long chain monomeric aliphatics, cuticular
55 waxes (Kolattukudy, 1980; Zeisler-Diehl et al., 2018). The cuticle reduces water loss,
56 provides protection against UV radiation, and is the primary interface for plant
57 microorganism and insect interactions (Riederer & Schreiber, 2001; Serrano et al., 2014;
58 Yeats et al., 2012) . The cutin is a biopolymer which consists mainly of ω – and midchain
59 hydroxy and epoxy fatty acids C_{16} - C_{18} as well as glycerol (Graça, 2002; Pollard et al., 2008;
60 Wattendorff & Holloway, 1980). The cutin forms the structural backbone of the cuticle as it is
61 known to prevent mechanical damage. The cuticular waxes are the second major
62 component of the leaf cuticle mostly consisting of alkanes, alcohols, acids, and aldehydes of
63 chain lengths between C_{16} - C_{32} . Cuticular waxes may also include secondary metabolites
64 such as flavonoids, triterpenoids and phenylpropanoids (Jeffree, 2006). Cuticular waxes can
65 be separated into two distinct waxes. The intracuticular wax within the cutin polymer is
66 clearly distinct from the epicuticular wax which is on the outer surface of the cutin polymer
67 (Buschhaus & Jetter, 2011; Samuels et al., 2008). These differences thus affect the physical
68 properties of the plant surfaces. The composition of the cuticular waxes is dependent on
69 plant species and environmental conditions (Jetter et al., 2006; Shepherd & Wynne Griffiths,
70 2006). Wax monomers are very energy rich and a potential source of energy and carbon if
71 they are bioavailable. However, it is still unclear if bacteria are able to utilise these aliphatic
72 compounds constituting the cuticle of living leaves as a source of carbon and if surfactants
73 would facilitate the utilisation.

74 Leaves are home to a manifold of bacteria and they can be covered by up to 5% bacterial
75 biomass (Remus-Emsermann et al., 2014; Schlechter et al., 2019). Many leaf surface

76 colonising genera were previously shown to degrade hydrocarbons, e.g. *Rhodococcus* spp.,
77 *Sphingomonas* spp., *Pantoea* spp., *Methylobacterium* spp., and Pseudomonads (Kertesz &
78 Kawasaki, 2010; Oso et al., 2019; Pizzolante et al., 2018; Salam et al., 2015).
79 Pseudomonads are common leaf colonisers and have many different ecological roles, e.g.
80 many *Pseudomonas syringae* strains can be bonafide and host specific pathogens (Xin et
81 al., 2018) while others may act as antagonists against agents of plant disease (Cabrefiga et
82 al., 2007; Zengerer et al., 2018) or have unknown, tritagonistic (Freimoser et al., 2016),
83 functions in the microbiota (Remus-Emsermann et al., 2016; Schmid et al., 2018).
84 Pseudomonads have the ability to produce so-called biosurfactants in common (D'aes et al.,
85 2010). Biosurfactants are biologically produced amphiphilic molecules consisting of a
86 hydrophilic head group and a hydrophobic moiety.
87 Leaf colonising Pseudomonads produce cyclic peptide biosurfactants (D'aes et al., 2010).
88 Their ecophysiological role is not always clear, but it has been shown that Pseudomonads
89 may gain different fitness advantages by producing surfactants including increasing survival
90 during fluctuating humidity conditions on leaves (Burch et al., 2014) and by increasing local
91 water availability due to the hygroscopic nature of their surfactants (Hernandez & Lindow,
92 2019). On agar plates it has been shown that biosurfactants increase surface mobility by
93 swarming and it has been assumed that they may serve similar functions on leaves (Lindow
94 & Brandl, 2003).
95 In this study, we characterised the physiological effect of biosurfactants in four different
96 Pseudomonads that were isolated from leaves of spinach (*Pseudomonas* sp. FF1) or
97 Romaine lettuce (*Pseudomonas* spp. FF2, FF3, and FF4) respectively. Their biosurfactants
98 were characterised using mass spectrometry and their physical properties were analysed.
99 Furthermore, we investigated the ecophysiological functions of the biosurfactants for the
100 bacteria. To that end, random insertion libraries were produced and biosurfactant knockout
101 mutants identified. The knockout mutants were characterised in a series of experiments that
102 investigated fitness changes *in vitro* and *in planta*.

103

104

105 **Material and Methods**

106 ***Bacterial strains used in this study***

107 Bacteria used in this study were *Pseudomonas* sp. FF1 (PFF1), *Pseudomonas* sp. FF2
 108 (PFF2), *Pseudomonas* sp. FF3 (PFF3), *Pseudomonas* sp. FF4 (PFF4) (Burch et al., 2011);
 109 All Pseudomonads were kind gifts of Adrien Burch and Steven Lindow (UC Berkeley)) and
 110 *E. coli* Stellar (Lucigen). PFF1 was isolated from spinach, PFF2, PFF3, and PFF4 were
 111 isolated from Romaine lettuce. Pseudomonads were routinely grown on liquid King's B (KB,
 112 20 g proteose peptone, 1.15 g K₂HPO₄, 1.5 g Mg[SO₄]*7H₂O. 10 g glycerol per liter, pH 7; for
 113 agar medium KBA, add 15 g agar per liter) or Lysogeny Broth (LB, 5 g yeast extract, 10 g
 114 tryptone, 10 g NaCl per liter, pH 7; for agar medium add 15 g agar per liter). *E. coli* was
 115 routinely grown on LB and LBA. For *in planta* competition experiments, spontaneous
 116 streptomycin resistant mutants of the wildtype Pseudomonads were selected (Newcombe &
 117 Hawirko, 1949). Where appropriate, the media were supplemented with kanamycin (50 µg
 118 ml⁻¹) or streptomycin (50 µg ml⁻¹).

119

120 ***16S rRNA gene sequencing***

121 To determine the phylogeny of the strains, their 16S rRNA gene was amplified from genomic
 122 DNA that was extracted using the NucleoSpin® Microbial DNA Kit (Macherey Nagel)
 123 following the manufacturer's recommendations. A PCR using KAPA2G Fast 2x Ready Mix
 124 with Dye (Kapa) was performed using the manufacturer's recommendation, 1 µL of genomic
 125 and 16S rRNA gene targeting primers SLK8-F 5'-AGAGTTTGATCATGGCTCAGAT-3' and
 126 SRK1506-R 5'-TACCTTGTTACGACTTCACCCC-3'. Resulting ~1.5 Kbp fragments were
 127 sequenced (Eurofins Genomic) and then curated and assembled using Geneious prime
 128 (Geneious). The assembled fragments were uploaded to ezbiocloud (Yoon et al., 2017) and
 129 the 30 best matches of organisms that were validly named were recovered for each of the
 130 four strains. Additional *Pseudomonas* 16S sequences and outgroup sequences were
 131 recovered from the silva database (Glöckner et al., 2017). All sequences were compiled into
 132 a fasta file and aligned and visualised using the FastME/OneClick option of ngphylogeny.fr
 133 (Lemoine et al., 2019). The resulting tree was imported into iTol, edited for publication and
 134 then exported (Letunic & Bork, 2019).

135

136 ***Preparation of electrocompetent Pseudomonads***

137 Electrocompetent Pseudomonads were produced as explained elsewhere (Artiguenave et
 138 al., 1997). Briefly, bacteria were grown overnight in 6 ml KB in a shaking incubator at 25 °C.
 139 Three ml of the overnight culture were then used to inoculate 100 ml KB that were incubated

140 at 25 °C in a shaking incubator until the culture reached mid-exponential growth phase
141 OD_{600nm} of approximately 0.6. The culture was then split in 50 ml aliquots and cooled on ice
142 for 30 minutes. Bacteria were then harvested by centrifugation at 6000 g and 4 °C for 10
143 minutes. The supernatant was dismissed and the aliquots were washed twice with 50 ml
144 ice-cold sterile water. Then they were washed in 25 ml ice-cold water and the aliquots were
145 combined again. After a final centrifugation, the cell pellet was resuspended in 250 µl sterile
146 10% glycerol and distributed in 50 µl aliquots that were stored at -80 °C.

147

148 ***Random transposon mutagenesis***

149 Random knockout mutants were produced using the EZ::Tn5Tm <KAN-2> Tnp
150 TransposomeTm kit (Epicentre) following the manufacturers recommendations. In brief, 50 µl
151 electrocompetent *Pseudomonads* were thawed on ice and 1 µl Tn5-transposome and 1 µl
152 endonuclease inhibitor were mixed with the cells. The mix was incubated for 5 minutes on
153 ice before the cells were pipetted into a pre-chilled 0.1 cm gap electroporation cuvette. A
154 gene pulser (Bio-Rad) was used to pulse the cells (2.5 kV, 200 Ω, 25 µF). Immediately after
155 that, 1 ml SOC (SOB: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 250 mM KCl per
156 liter, pH 7. SOC: SOB supplemented with 5 ml 2 M MgCl₂ and 20 ml 1 M glucose) was
157 added and the cells were incubated for 1 hour at 30 °C and 150 rpm. Transposon insertion
158 mutants were selected on minimal medium agar plates (15 ml glycerol, 5 g L-glutamin, 1.5 g
159 K₂HPO₄, 1.15 g MgSO₄ × 7H₂O, 15 g agar per liter, pH 7) supplemented with kanamycin.
160 Minimal medium was used to prevent the growth of auxotrophic mutants. Transposon
161 mutants could be detected after 2 days.

162 To determine the site of transposon integration, genomic DNA of knockout mutants was
163 isolated using the ISOLATE II kit (Bioline). Genomic DNA was cut using KpnI (New England
164 Biolabs) or EcoRI and ligated into similarly digested and dephosphorylated vector pUC19
165 (New England Biolabs) using T4-ligase (New England Biolabs) following the
166 recommendations of the manufacturer. 5 µl per ligation mix were transformed into chemical
167 competent *E. coli* Stellar using the manufacturers recommendations. Clones harboring
168 plasmids containing the transposon were selected on LB supplemented with kanamycin.
169 Inserts of the plasmids were sequenced using the transposon specific primer kan2_RP-1
170 (5'-gcaatgtaacatcagagatttgag-3'). Sequencing results were compared to the NCBI database
171 using NCBI BLAST restricted to the genus *Pseudomonas* (Altschul et al., 1990).

172

173 ***Screens for surfactant production***

174 To screen for surfactant production, the atomised oil assay was performed (Burch et al.,
175 2010). To that end, agar plates containing transposon mutants were sprayed with
176 hydrophobic dodecan using an airbrush. Bacterial colonies that produced surfactants
177 resulted in a halo around the colony where the surfactant in the agar changes the surface
178 angle of oil droplets on the surface. Colonies that lacked this characteristic halo were further
179 characterised. Presumptive surfactant mutants were tested in the drop collapse assay as
180 described previously (Oso et al., 2019). Briefly, 2 µl of Magnatec 10W-40 oil (Castrol) were
181 pipetted into each well of a 96-well plate lid (Corning incorporated) and were allowed to
182 equilibrate for 2 hours to ensure that each well was evenly coated. Bacterial overnight
183 cultures were centrifuged at 2600 × g for 10 minutes. Five µL of the culture supernatant was
184 pipetted into the centre of an oil filled well. Drops that collapsed into the oil, i.e. decreased
185 their contact angle, were positive for surfactant production while drops that remained intact
186 and stayed on top of the oil were negative for surfactant production. All experiments were
187 performed in at least 8 biological replicates.

188

189 ***Extraction of surfactants***

190 Bacterial strains were grown as crude streaks on five separate KBA plates for 48 hours at
191 25 °C. Afterwards, bacterial biomass was harvested using 5 ml of sterile water per plate and
192 the cell suspensions of all 5 plates were combined in a 50 ml centrifugation tube. 25 ml ethyl
193 acetate was added to the suspension and the tube was vortexed for 3 minutes. The mixture
194 was then centrifuged for 10 minutes at 1000 x g to facilitate separation of the aqueous and
195 organic phase. The organic phase was recovered using a glass pipette and transferred to a
196 glass vessel before the ethyl acetate was evaporated off under constant nitrogen flow. The
197 result was resolved in ethanol and sterile filtered through a 0.22 µm filter. The filtered
198 solution was then dried under constant nitrogen flow and weight before it was resuspended
199 to 5 µg ml⁻¹ in ethyl acetate.

200

201 ***Mass-spectrometric analysis***

202 Mass spectrometric analysis of the biosurfactants was performed using a QTRAP 4500
203 (Applied Biosystems, AB Sciex) triple-quadrupole mass spectrometer, operated in negative
204 electrospray ionization (ESI) – Q1 Scan Modus. The surfactant solution with a concentration
205 of 5 µg ml⁻¹ was injected via a syringe pump set to a flow rate of 10 µl min⁻¹ directly into the
206 MS. The analytes were detected in negative mode within a mass over charge range of 1000
207 – 1200 m/z.

208

209

210 **Plant growth and in planta experiments**

211 *Arabidopsis thaliana* was grown axenically as described previously (Miebach et al., 2020).
 212 Briefly, *Arabidopsis* seeds were sterilised in a 1.5 ml Eppendorf tube by adding 1 mL 70 %
 213 ethanol and 0.1 % Triton X-100. The seeds were vortexed and then incubated for one
 214 minute. The supernatant was removed by pipetting, followed by the addition of 1 ml 10 %
 215 bleach and 10 µl of 0.1 % Triton X-100 for 12 minutes. After removing the bleach, the seeds
 216 were rinsed thrice with 1 ml of sterile distilled water were stratified for 48 hours at 4 °C.
 217 Stratified seeds were pipetted onto Murashige and Skoog-agar (MS-agar, 2.2 g of Murashige
 218 and Skoog medium including vitamins (Duchefa) and 10 g plant agar (Duchefa) per litre of
 219 milliQ water, pH 5.8) filled 200 µL pipette tips that were shortened by 1 cm to allow the
 220 plant's roots to easily pass the tip. The tips were placed pointy end first into a MS-agar plate.
 221 The seeds were germinated for seven days at short day conditions (11 hours day/ 13 hours
 222 night). After the germination period, the seedling-filled tips were transferred to autoclaved
 223 Magenta™ GA-7 (bioWORLD) plant culture boxes filled with finely ground 90 g zeolite clay
 224 (Purfit Clay Litter, Vitapet) and 60 ml MS medium. Four seedlings were transferred into each
 225 Magenta box and the plants were grown for an additional three weeks at short day
 226 conditions (11 hours day/ 13 hours night, chamber set to 85% relative humidity). To prepare
 227 bacterial inoculum, bacteria were cultured on LB broth overnight. Bacteria were then
 228 harvested by 10 min centrifugation at 2600 g and washed with 1 × phosphate buffer saline
 229 (PBS, 0.2 g L⁻¹ NaCl, 1.44 g L⁻¹ Na₂HPO₄ and 0.24 g L⁻¹ KH₂PO₄). Bacteria were
 230 resuspended to an OD_{600nm} 0.5 and then serial diluted to OD_{600nm} 0.00005. For competition
 231 experiments wildtype and surfactant knockout strains were mixed at a ratio of 1:1. 100 µL of
 232 the mix or the monocultures were inoculated onto three week-old *Arabidopsis* using an
 233 T-180 airbrush (KKmoon).

234 Bacteria were recovered by harvesting the leaf material of individual plants, placing them in
 235 a 1.5 ml Eppendorf vial. The plants were weighed and 1 mL 1 × PBS were added. The vial
 236 was vortexed for 2 minutes and then sonicated for 5 minutes in a sonication bath
 237 (Elmasonic) before they were vortexed for another 2 minutes. The supernatant was serial
 238 diluted and CFU of wildtype and surfactant mutants were determined by growing the strains
 239 on LB agar containing appropriate antibiotics to select for either the spontaneous
 240 streptomycin resistant wildtype or the kanamycin resistant mutants.

241

242 **Diesel utilisation assay**

243 To measure the ability of wildtype and surfactant knockout mutants to grow on diesel as the
 244 sole source of carbon, Bushnell-Haas broth (0.2 g L⁻¹ MgSO₄, 0.02 g L⁻¹ CaCl₂, 1.0 g L⁻¹

245 KH_2PO_4 , 1.0 g L^{-1} K_2HPO_4 , 1.0 g L^{-1} NH_4NO_3 and 0.05 g L^{-1} FeCl_3 , pH 7.2), was
 246 supplemented with 1% diesel (commercial diesel, locally sourced) (Oso et al., 2019).
 247 Bushnell-Haas broth without additional carbon source was used as a negative control. In
 248 control experiments, to complement surfactant knockout mutants, between 0.23-0.265 mg
 249 mL^{-1} of isolated WT surfactants or 0.1 mg mL^{-1} Tween-20 were supplemented. Bacteria were
 250 grown overnight in LB, diluted $100 \times$ using Bushnell-Haas broth without carbon source. The
 251 diluted bacterial suspensions were inoculated into 50 mL broth cultures in 250 mL
 252 Erlenmeyer flasks. Cultures were incubated at 30°C and 200 rounds per minutes for up to 17
 253 days. Cell density was regularly measured by determining the optical density at 600 nm
 254 using a spectrophotometer (Biochrom WPA CO8000, Biowave). All experiments were
 255 performed in three biological replicates.

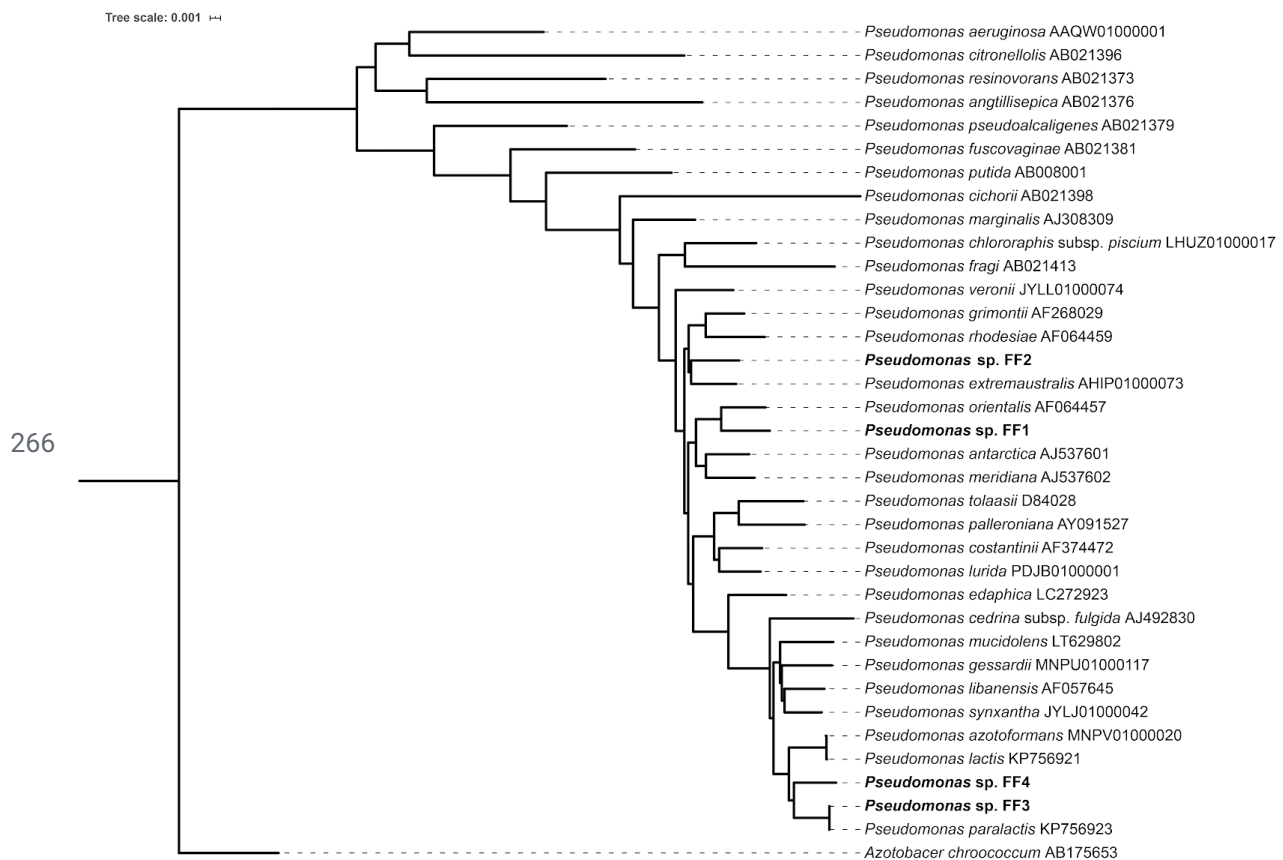
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257 Results

258 *Phylogenetic placement of Pseudomonas sp. FF1, FF2, FF3 and FF4*

259 Analysis of the 16S rRNA genes of all four isolates revealed that they are all members of the
260 genus *Pseudomonas* and members of the *Pseudomonas fluorescens* lineage and subgroup
261 (Peix et al., 2018). PFF1 clusters closely with *Pseudomonas orientalis*, PFF2 clusters closely
262 with *Pseudomonas extremaustralis*, while PFF3 and PFF4 cluster closely with *Pseudomonas*
263 *paralactis* (Figure 1). PFF1 and PFF2 are closer related to each other than to PFF3 and
264 PFF4. PFF3 and PFF4 are closely related.

265



267 **Figure 1. Phylogenetic placement of the four isolated Pseudomonads.** The newly sequenced
268 isolates are highlighted in bold. NCBI accession numbers of the respective sequences are noted
269 behind the species names. *Azotobacter chroococcum* was used as an outgroup.

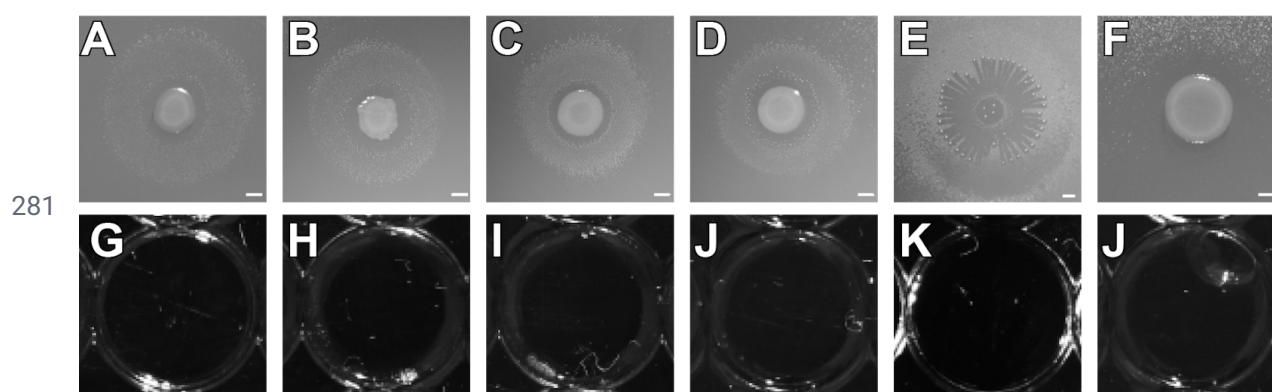
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271 *Surfactant production of tested Pseudomonads*

272 All four wildtype *Pseudomonads* were tested for their production of surfactants on agar
273 plates using the atomised oil assay (Burch et al., 2010; Oso et al., 2019). All four strains
274 produced clear halos where the reflection of the oil to light changed indicating production of
275 surfactants (Figure 2A-D). Similarly, the positive control Tween-20 showed a halo (Figure
276 2E), while the negative control, *E. coli* Dh5α, was lacking a halo (Figure 2F). The drop
277 collapse assay was used as a secondary test for surfactant production. All tested wildtype

278 culture supernatants collapsed into the engine oil (Figure 2G-J). The collapse is due to a
279 change in surface tension of the supernatant.

280



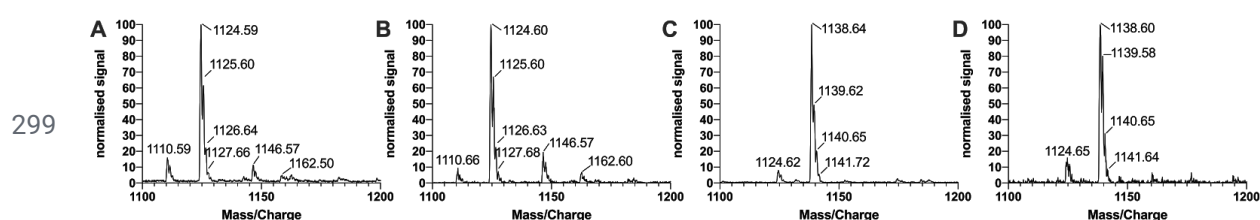
282 **Figure 2. A-F) Atomised oil assays to demonstrate the production of surfactants. A-D)** wildtype
283 colonies of *PFF1*, *PFF2*, *PFF3*, and *PFF4*, respectively, exhibiting a halo indicative for surfactant
284 production. **E)** Tween-20 **F)** *E. coli* Dh5α **G-L) Drop collapse assays to demonstrate the**
285 **production of surfactants.** Culture supernatants of wildtype *PFF1*, *PFF2*, *PFF3*, and *PFF4*,
286 respectively, collapsed into oil indicative for surfactant production. **K)** collapsed drop containing
287 Tween-20. **L)** Non-collapsed drop if *E. coli* culture supernatant.

288

289 Mass spectrometric analysis surfactants

290 The analysis of surfactants harvested from the *Pseudomonads* using LC-MS with ESI in
291 negative mode revealed that *PFF1* and *PFF2* produced the same compounds with a
292 characteristic main peak at $m/z=1124.59$, which can be attributed to the deprotonated
293 molecular ion $[M-H]^-$. The analogous pattern for the protonated molecular ion $[M+H]^+$ has
294 been previously described for the cyclic lipopeptide viscosin when using ESI in positive
295 mode for detection (de Bruijn et al., 2008; Laycock et al., 1991). Similarly, *PFF3* and *PFF4*
296 share the same characteristic main peak at $m/z=1138.60$, the analogous pattern has
297 previously been described for the cyclic lipopeptide massetolide A (de Bruijn et al., 2008).

298



300 **Figure 3 A-D) MS/MS spectra of extracted surfactants of *PFF1*, *PFF2*, *PFF3*, and *PFF4***
301 **respectively. *PFF1* and *PFF2* both produce viscosin, *PFF3* and *PFF4* both produce massetolide A.**
302 **Spectra were normalised against the maximal intensity.**

303

304

305

306 **Random Tn5 mutagenesis and mutant characterisation**

307 The surfactants producing wildtypes were subjected to random insertion mutagenesis using
 308 the EZ-Tn5 transposon system. The screen resulted in a transposon mutant library with
 309 several hundred transposon mutants for each of the four isolates. Each of the mutant
 310 libraries was screened with the atomised oil assay for lack of surfactant production mutants.
 311 Mutants lacking surfactant production were identified and one mutant for each isolate was
 312 selected for further characterisation (Figure 4A-D). The drop collapse assay was conducted
 313 and confirmed the results of the atomised oil assay (Figure 4E-H). The insertion site of each
 314 mutant was determined by digesting the genomic DNA of the mutants and cloning it into
 315 pUC19 before selecting for kanamycin resistance encoded in the transposon (Supplemental
 316 Table 1).

317 The investigated *PFF1* mutant carried an insertion in a gene with 97% similarity to a
 318 non-ribosomal peptide synthetase in *P. orientalis* F9 (Genbank: BOP93_14875) (Zengerer et
 319 al., 2018) which has an 80% peptide similarity to the *viscB* gene of *P. fluorescens* SBW25
 320 (UniProtKB ID: C3K9G2) (De Bruijn et al., 2007; Silby et al., 2009). The investigated *PFF2*
 321 mutant carried an insertion in a gene with a 86% similarity to the *viscB* gene (Genbank:
 322 CAY48788.1) of *P. fluorescens* SBW25, respectively. Therefore, they are designated
 323 *PFF1::ezTn5-viscB* and *PFF2::ezTn5-viscB*, respectively. The *viscB* gene encodes for a
 324 non-ribosomal peptide synthetase that, in conjunction with *viscA* and *viscC*, produces the
 325 cyclic lipopeptide biosurfactant viscosin (De Bruijn et al., 2007). The *PFF3* Tn5 transposon
 326 mutant carried an insertion in a gene with 99% similarity to the *massB* gene in
 327 *Pseudomonas fluorescens* SS101 (Genbank: ABH06368.2). The *PFF4* Tn5 transposon
 328 mutant carried an insertion in a gene with 95% similarity to the *massB* gene in
 329 *Pseudomonas fluorescens* SS101 (de Bruijn et al., 2008). The *massB* gene is part of the
 330 massetolide A synthesis gene cluster. Therefore the mutants were designated
 331 *PFF3::ezTn5-massB* and *PFF4::ezTn5-massB*.

332

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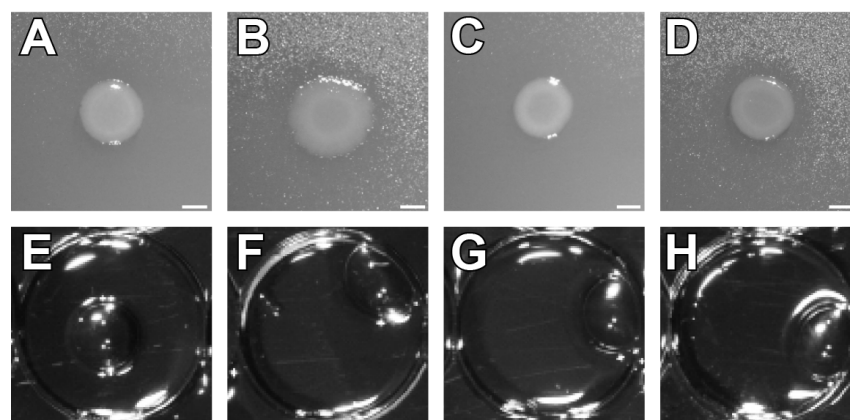


Figure 4. A-D) Atomised oil assay to demonstrate the production of surfactants. Tn5-transposon insertion mutant colonies *PFF1::ezTn5-visB*, *PFF2::ezTn5-visB*, *PFF3::ezTn5-massB*, and *PFF4::ezTn5-massB*, respectively, lacking a halo indicative for surfactant production. **E-H) drop collapse assays to demonstrate the production of surfactants.** Culture supernatants of Tn5-transposon insertion mutant *PFF1::ezTn5-visB*, *PFF2::ezTn5-visB*, *PFF3::ezTn5-massB*, and *PFF4::ezTn5-massB*, respectively, showing a beaded bubble swimming on top of oil, indicative for the lack of surfactants.

After extracting agar plates to recover surfactants for the analysis using mass spectrometry, no surfactants could be detected (Figure 5 A-D).

The effect of the transposon insertions and the lack of surfactant production was tested in shaking liquid cultures in two different conditions, either KB complex medium (Supplemental Figure 1A), or M9 minimal medium supplemented with glucose as the sole source of carbon (Supplemental Figure 1B). None of the tested insertion mutants exhibited significantly changed doubling times under the two tested conditions.

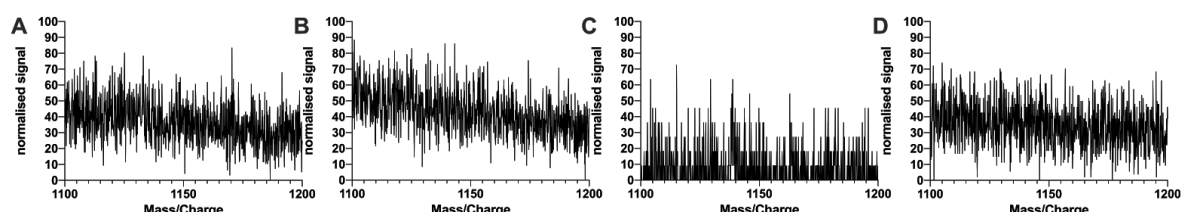
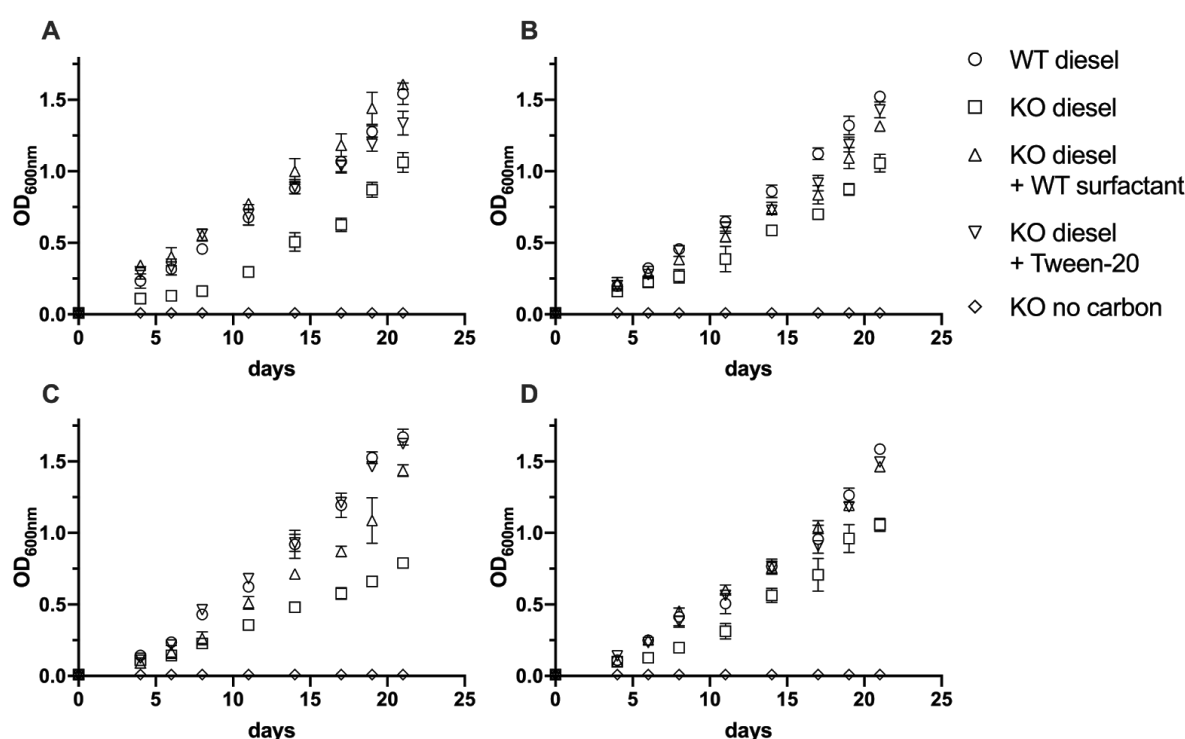


Figure 5 A-D) Knockout mutants show no sign of surfactant production - MS/MS spectra of extracts of *PFF1::ezTn5-visB*, *PFF2::ezTn5-visB*, *PFF3::ezTn5-massB*, and *PFF4::ezTn5-massB*, respectively. None of the random knockout mutants produced detectable surfactant peaks at the respective wildtype m/z values. Spectra were normalised against the maximal intensity.

360 **Growth on diesel oil as sole carbon source**

361 To investigate if the lack of surfactant production could impact the ability of the
 362 *Pseudomonad* strains to degrade alkanes, the different wildtypes and transposon mutants
 363 were grown on Bushnell-Haas broth with diesel as the sole carbon source. This experiment
 364 revealed that all surfactant mutants, even though they were still able to grow on diesel, had a
 365 reduced growth rate, and a reduced final optical density after up to 21 days of growth (Figure
 366 6). No growth could be observed on Bushnell-Haas broth without carbon source for either
 367 the wildtype or the surfactant mutants (data not shown). In general, the growth on diesel oil
 368 was slower compared to growth on complex medium or minimal medium supplemented with
 369 glucose as sole carbon source and better described by a linear function than an exponential
 370 growth function. By supplementing knockout mutants growing on diesel with biosurfactants
 371 harvested from respective wildtype strains or Tween-20 growth on diesel could be
 372 complemented in parts or completely compared to the wildtype. Knockout mutants could not
 373 grow on provided surfactants to a degree that explains the increased growth on diesel
 374 (Supplemental figure 2).

375

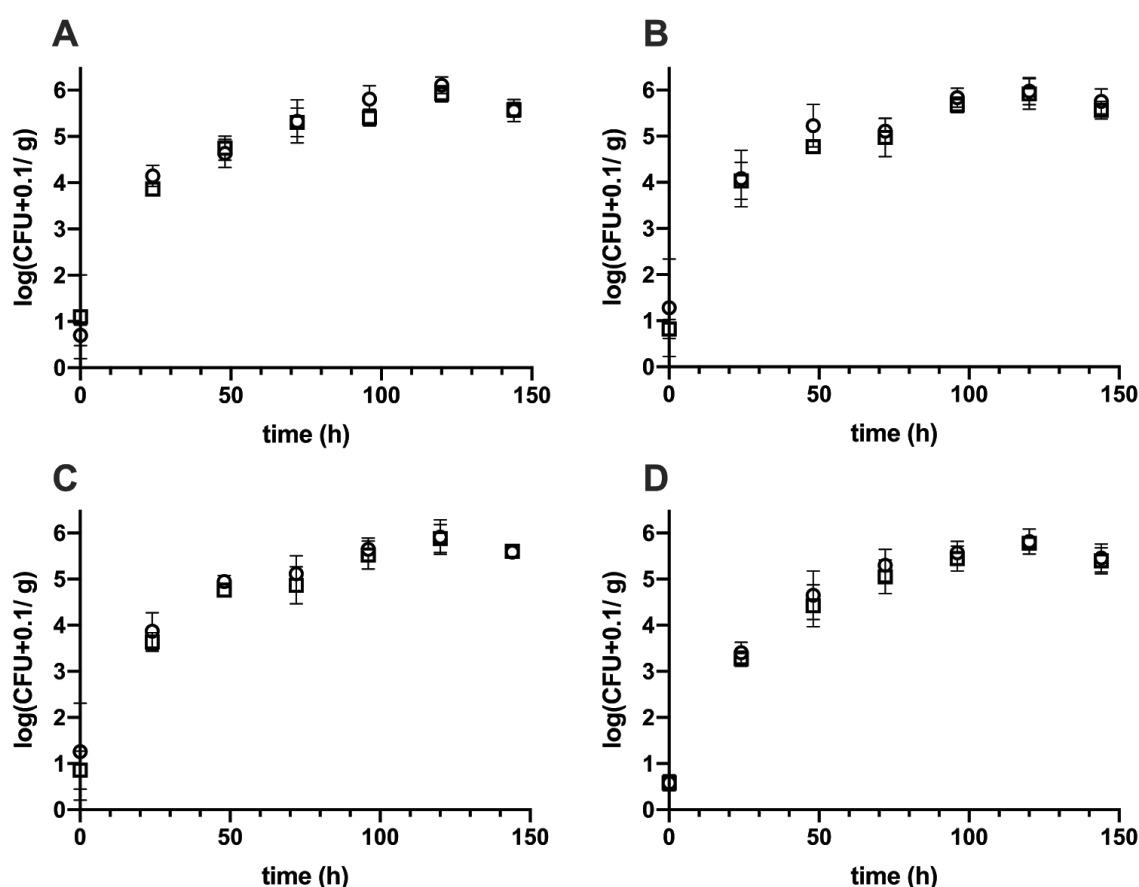


377 **Figure 6. Utilisation of diesel by biosurfactant knockout mutants and wildtypes.** A) PFF1, B)
 378 PFF2, C) PFF3, D) PFF4. Each wildtype and knockout mutant was grown in Bushnell-Haas broth
 379 supplemented with diesel as the sole source of carbon (circle and square, respectively). Knockout
 380 mutants were complemented with either wildtype surfactant (triangle), Tween-20 (inverted triangle) or
 381 were incubated with no additional carbon source (diamond). Error bars depict the standard deviation
 382 of the mean.

383

384 **Fitness in planta**

385 To investigate changes in the ability of the transposon mutants to colonise leaf surfaces, the
386 mutants were co-inoculated with the respective wildtypes by airbrushing. Whole
387 above-ground plant material was sampled daily for six days and colony forming units of
388 wildtype and transposon mutants were determined (Figure 7). The initial bacterial densities
389 were similar between wildtype and knockout mutants. wildtypes (PFF1, PFF2, PFF3 and
390 PFF4) and corresponding mutants (PFF1::ezTn5-viscB, PFF2::ezTn5-viscB,
391 PFF3::ezTn5-massB and PFF4::ezTn5-massB) colonised *Arabidopsis* at similar rates. PFF1,
392 PFF2 and their mutants reached approximately 10^7 CFU per gram of plant weight, whereas
393 PFF3, PFF4 and their mutants reached approximately 10^6 CFU per gram of plant weight.
394 Thus, no differences between the plant colonisation of wildtype and mutants were found.
395 Furthermore, growth in planta of all strains was tested individually, no significant differences
396 in plant colonisation could be determined (Supplemental figure 3).



397

398 **Figure 7. In planta competition of wildtypes (open circles) and mutants (open squares).** A)
399 PFF1 vs. PFF1::ezTn5-viscB, B) PFF2 vs. PFF2::ezTn5-viscB, C) PFF3 vs. PFF3::ezTn5-massB, D)
400 PFF4 vs. PFF4::ezTn5-massB. wildtypes are depicted by circles, knockout mutants by squares. Error
401 bars depict the standard deviation of the mean.

402

403 Discussion

404 All four *Pseudomonads* isolated from either spinach or romaine lettuce leaf material (Burch
405 et al., 2016) belong to the fluorescent *Pseudomonads* (Gomila et al., 2015). *PFF1* and *PFF2*
406 are phylogenetically more closely related to each other than to *PFF3* and *PFF4*. *PFF3* and
407 *PFF4* are very closely related. All four strains are produced surfactants on agar plates and in
408 liquid culture as shown by the atomised oil assay and the drop collapse assay. As the ability
409 to produce surfactants is widely distributed in the genus *Pseudomonas*, this result was not
410 surprising (Geudens & Martins, 2018; Nybroe & Sørensen, 2004). The relatedness of the
411 strains is also reflected in the surfactants that each of the strains is producing: *PFF1* and
412 *PFF2* produce the viscosin-like surfactants, while *PFF3* and *PFF4* are produced massetolide
413 A-like surfactants. The production of viscosin and massetolide A by *Pseudomonads* has
414 been demonstrated previously (de Bruijn et al., 2008). Both viscosin and massetolide A are
415 the product of nonribosomal peptide synthetase genes. Viscosin production depends on a
416 gene cluster encompassing the three genes *viscA*, *viscB*, and *viscC* and which spans
417 approximately 32 kb (De Bruijn et al., 2007). Massetolide A production also depends on a
418 gene cluster which encompasses the three genes *massA*, *massB* and *massC* and spans
419 approximately 30 kb (de Bruijn et al., 2008).

420 To further investigate the ecological function of the surfactants in the leaf colonising
421 *Pseudomonads*, random Tn5 transposon insertion mutants were produced and further
422 characterised. The screen yielded complete loss of surfactant production mutants for every
423 strain, indicating that each strain only encodes for one surfactant that is active during the
424 selection conditions. The insertion sites were mapped to genes that matched previously
425 characterised non-ribosomal peptide synthase clusters responsible for surfactant production,
426 and which matched the surfactants that were identified using mass-spectrometry. *PFF1* and
427 *PFF2* knockout mutants were mapped to *viscB* gene homologues, and *PFF3* and *PFF4*
428 knockout to *massB* gene homologues (De Bruijn et al., 2007; de Bruijn et al., 2008).

429 The assumption that only one surfactant is produced by each strain was corroborated by a
430 sequence of experiments during which the surfactant mutants consistently failed to produce
431 signs of surfactant production independent of their growth conditions. The surfactant mutants
432 failed to produce halos in the atomised oil assay, and the culture supernatant did not
433 collapse into motor oil in the drop collapse assay. Mass spectrometric analysis of the
434 knockout mutants showed that the production of surfactants was completely abolished and
435 no detectable peak pattern was found after the surfactant extraction procedure (Figure 5).

436 Despite the loss of surfactant production and the additional burden of expressing the
 437 kanamycin resistance gene from the Tn5 transposon, the insertions had no detectable
 438 fitness effects in either complex KB medium or minimal M9 medium supplemented with
 439 glucose. In shaking liquid cultures, surfactants did not provide critical functions for growth
 440 (Supplemental figure 1). We hypothesise that surfactants may enable bacteria to utilise parts
 441 of the plant cuticle as a source for carbon. Even though it was not possible to show that
 442 *Pseudomonads* and their respective mutants had differential abilities to utilise hydrocarbon
 443 components from isolated cuticles (data not shown), a clear difference in the ability of
 444 wildtype and mutant to utilise diesel for growth was demonstrated (Figure 6). Even though
 445 growth was not completely abolished, it was markedly reduced. This could also explain why
 446 growth on isolated cuticles did not yield conclusive results and differences between wildtype
 447 and knockout mutant. Due to the size of the non-ribosomal peptide synthetase genes, it was
 448 not possible to construct rescue mutants. However, we attempted to complement the
 449 reduced ability of the knockout mutants to degrade diesel oil by adding harvested wildtype
 450 surfactant or Tween-20 to growing cultures. Indeed, both surfactants were able to
 451 complement the growth phenotype either in parts or completely, evidencing that the lack of
 452 surfactants was the causal reason for reduced growth. Despite the chain length differences
 453 between the diesel (Wante & Leung, 2018) and the alkane monomers in waxes of leaf
 454 cuticles (Zeisler-Diehl et al., 2018), the chemistry of both aliphatic mixtures contain similar
 455 monomers. It is thus not unthinkable that, under nutrient limiting conditions, the
 456 *Pseudomonas* strains tested here are able to utilise aliphatic components of leaf cuticles in a
 457 surfactant-dependent manner. However, we failed to provide a final proof of this relationship.
 458 To investigate the role of the surfactants during plant colonisation we inoculated axenically
 459 grown *Arabidopsis* with mixtures of wildtype and knockout mutants or with individual strains.
 460 During co-inoculation with their respective wildtypes onto axenic *Arabidopsis*, no fitness
 461 disadvantages for the knockout mutants were detected. This might be a consequence of the
 462 surfactant acting as a public good that increases the fitness of wildtype and co-inoculated
 463 mutants alike (Lyons & Kolter, 2017). However, single strain inoculations also did not result
 464 in a diminished ability of the knockout mutants to colonise *Arabidopsis*. This is in contrast to
 465 previous experiments that demonstrated that surfactants do indeed have a positive effect on
 466 plant colonisation (Burch et al., 2014). It is noteworthy that the experimental setup used in
 467 our study was markedly different including a different plant host as well as incubation
 468 conditions under constant relative humidities. While previously it was shown that fluctuating
 469 humidities are a prerequisite to result in a fitness advantage. Therefore, it might still be

470 possible the surfactants in the here-tested strains will impact plant colonisation for example
471 under fluctuating relative humidities.

472

473 **Conclusion**

474 The experiments reported here demonstrated that the biosurfactants produced by four
475 different leaf colonising *Pseudomonads* impacted on their ability to degrade aliphatic
476 compounds. However, the ability to produce biosurfactants had no measurable impact on the
477 ability of the strains to colonise axenic *Arabidopsis* leaves in competition or after individual
478 strain inoculations. We gathered additional evidence that the bacteria may utilise aliphatic
479 compounds originating from leaf cuticles but failed to conclusively demonstrate a relationship
480 between surfactant production and leaf colonisation ability. Future studies will have to be
481 performed to address this hypothesis.

482

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487

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