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N-Acyl Homoserine Lactones and Lux Solos Regulate Social Behaviour and Virulence of *Pseudomonas syringae* pv. *actinidiae*

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1 [N-acyl homoserine lactones and Lux solos regulate social behaviour and](#)  
2 [virulence of \*Pseudomonas syringae\* pv. \*actinidiae\*](#)  
3

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24

25 **CONFLICT OF INTEREST**

26 The authors declare that they have no conflict of interest.

27

28 ABSTRACT

29

30 The phyllosphere is a complex environment where microbes communicate through signalling  
31 molecules in a system, generally known as quorum sensing (QS). One of the most common QS system  
32 in Gram-negative proteobacteria is based on the production of N-acyl-homoserine lactones (AHLs)  
33 by a LuxI synthase and their perception by a LuxR sensor.

34 *Pseudomonas syringae* pv. *actinidiae* (Psa), the aetiological agent of the bacterial canker of kiwifruit,  
35 colonises plant phyllosphere before penetrating via wounds and natural openings. Since Psa genome  
36 encodes three LuxR solos without a cognate LuxI, this bacterium may perceive diffusible signals, but  
37 it cannot produce AHLs, displaying a non-canonical QS system. The elucidation of the mechanisms  
38 underlying the perception of environmental cues in the phyllosphere by this pathogen and their  
39 influence on the onset of pathogenesis are of crucial importance for a long-lasting and sustainable  
40 management of the bacterial canker of kiwifruit.

41 Here, we report the ability of Psa to sense its own population density and the presence of surrounding  
42 bacteria. Moreover, we show that Psa can perceive AHLs, indicating that AHL-producing  
43 neighbouring bacteria may regulate Psa virulence in the host.

44 Our results suggest that the ecological environment is important in determining Psa fitness and  
45 pathogenic potential. This opens new perspectives in the use of more advanced biochemical and  
46 microbiological tools for the control of bacterial canker of kiwifruit.

47

48 **Keywords:** Bacterial canker, kiwifruit, quorum sensing, *Actinidia chinensis*, AHL

## 49 INTRODUCTION

50

51 The phyllosphere is a complex ecosystem, in which the host plant provides the primary source of  
52 nutrients (exudates, cell wall derivatives) supporting the survival of the epiphytic microflora.  
53 However, the phyllosphere is an inhospitable, oligotrophic and competitive environment, where  
54 microorganisms must adapt to sudden and drastic changes in environmental conditions and to limited  
55 and scattered resources [1]. In these harsh conditions, the competition among microbial species could  
56 be very high and lead to mutual exclusion [2,3]. On the other hand, the formation of symbiotic  
57 consortia, for instance through the reciprocal exchange of metabolites, is another strategy to overcome  
58 these limitations [4-6]. Thus, epiphytic microorganisms form complex communities, including plant  
59 symbionts, commensals, pathogens and opportunists, where the action of each individual species  
60 strictly depends on the network of ecological interactions inside and between each microbial  
61 population [3]. For instance, a pathogen might express its virulence only when the microbial  
62 composition of the surrounding communities is favourable [7,8]. The study of ecological relationships  
63 within multispecies communities has therefore become an emerging issue in plant-microbe  
64 interaction and plant pathology. One of the main factors coordinating the dynamics within microbial  
65 communities is the production, perception and response to signals among bacterial cells of a same  
66 species or belonging to different species. Several microbial communication systems, both intra- and  
67 interspecies, mediated by signals of different chemical natures, have been discovered and studied so  
68 far [9]. In Gram-negative proteobacteria, N-acyl homoserine lactones (AHLs) represent the most  
69 common signals mediating quorum sensing (QS) responses, i.e. the regulation of bacterial behaviour  
70 through modulation of gene expression in response to population density [10,11]. Signal specificity  
71 of AHLs is determined by the nature of their acyl moiety, i.e. the length of its carbon chain and the  
72 substitution at position C3. The archetypical AHL-QS system is based on a LuxI-LuxR protein pair,  
73 where the LuxI synthase is responsible for AHL biosynthesis and the LuxR receptor acts as a  
74 transcriptional regulator upon AHL binding [12]. LuxI/LuxR-encoding genes are usually adjacently

75 located, forming operons in bacterial genomes. Several LuxI/LuxR pairs can also co-exist in a single  
76 genome, leading to a real hierarchical QS network for bacterial behaviour control [13]. However,  
77 additional QS-type LuxR homologues have been identified, which lack a cognate LuxI, and were thus  
78 termed LuxR ‘solos’ [14,15]. These receptors, largely present in bacteria, might respond to AHLs  
79 released by other species in the environment [15], other diffusible compounds, such as pyrones [16],  
80 biosurfactants [17] and volatile compounds [18], or even eukaryotic compounds, including plant  
81 signal molecules in the case of plant-associated bacteria [19]. In the latter case, LuxR solos would  
82 not play a role in QS *per se* but rather in interkingdom communication between bacteria and their  
83 host plant.

84 *Pseudomonas syringae* pv. *actinidiae* (Psa) is the aetiological agent of kiwifruit (*Actinidia* spp.)  
85 bacterial canker. Psa was firstly isolated in Japan [20], but it started raising serious phytosanitary  
86 concerns since the pandemic outbreak of 2008, caused by a genetically separate lineage of Psa, termed  
87 biovar 3 [21,22]. In conducive conditions, the pathogen can cause plant death within one season from  
88 infection [23].

89 Before invading host tissues and spreading systemically, Psa grows epiphytically on asymptomatic  
90 kiwifruit plants [24,25]. Moreover, even after systemic infection, the host plant may remain  
91 asymptomatic or show only mild symptoms [22,26]. This phenomenon could be explained by  
92 environmental signals being perceived by Psa to regulate its own lifestyle, i.e. enhancing Psa survival  
93 and competition in the phyllosphere, or triggering its pathogenicity.

94 It was recently reported that Psa does not possess a canonical LuxI/LuxR QS system [27] but displays  
95 three putative LuxR solos (designated as PsaR1, 2, 3). Among them, PsaR2 was predicted to bind an  
96 unidentified plant-derived signal, while PsaR1 and PsaR3 showed some responsiveness to AHLs in  
97 an *in-vitro* assay, and may thus respond to AHLs produced by neighbouring bacteria [27].

98 The release of volatile compounds by Psa and their biological effects on kiwifruit plants have been  
99 investigated previously [28]. In contrast, Psa responses to airborne signals are less known. The semi-

100 volatile 1-undecene is one of the main compounds released by Psa and several other *Pseudomonas*  
101 species [29,30], but no signalling function has been described for it [31,32].  
102 This work examined the social behaviour and some virulence traits of Psa in response to the  
103 microflora present in *Actinidia* phyllosphere. The induction of bacterial motility and biofilm  
104 formation, which both contribute to epiphytic colonisation, was observed *in vitro*, together with the  
105 regulation of the expression of genes involved in these processes. Moreover, the effect on Psa fitness  
106 was also evaluated *in planta*, in terms of bacterial growth under controlled conditions. The  
107 experiments aimed at determining (i) the ability of Psa to perceive its own population density or (ii)  
108 the presence of other epiphytic bacteria; (iii) the role of AHLs and 1-undecene in mediating bacterial  
109 communication; (iv) the role of Psa LuxR solos in the perception of bacterial signals.  
110

## 111 MATERIALS AND METHODS

112

### 113 *Bacterial species, culture conditions and bacteria quantification*

114 The bacterial strains used in this work were: Psa strain CFBP7286, *P. syringae* pv. *syringae* strain  
115 ICMP849, *P. viridiflava* (isolated from *A. chinensis* var. *deliciosa* during this project) and *P.*  
116 *fluorescens* strain A506. All strains were grown in liquid Luria-Bertani (LB) medium at 27 °C under  
117 moderate shaking (120 rpm). The production of AHLs was assessed using *Chromobacterium*  
118 *violaceum* strain CV026 (sensitive to AHLs with a C8 acyl group or shorter) and *Agrobacterium*  
119 *tumefaciens* strain NT1 (pZLR4) (responding to a broad array of AHLs) [33]. The mutants Psa-mR1,  
120 Psa-mR2 and Psa-mR3 (carrying a knock-out mutation of the *luxR* solos *psaR1*, 2 or 3, respectively  
121 [27]) have also been used in this study.

122 Population densities during logarithmic bacterial growth in liquid cultures were determined by  
123 measuring their optical density at  $\lambda = 600$  nm (OD<sub>600</sub>) and confirmed by counting the number of  
124 colony forming units (CFU), after plating 10-fold serial dilutions of the bacterial culture on LB-agar  
125 medium. A standard curve of correspondence between OD<sub>600</sub> and population density assessed by  
126 plate counts was produced for each strain prior to experiments.

127

### 128 *Selection of target genes*

129 Genes were selected based on their potential regulation through QS mechanisms, putative role in cell  
130 density response and/or relevance to social phenotypes: bacterial motility (*fliP*, *pilA*, *pilC*, *pilO*),  
131 biofilm formation (*algD*, *wspR*, *wssB*), virulence effectors (*avrPto1*, *hopD1*, *hopS2*, *hopZ5*),  
132 biosurfactant production (*rhlA*, *syfA*) and quorum sensing (*psaR1*, 2, 3) (Supporting information  
133 Table S1). A TBLASTN search was performed with the amino acid sequences of the corresponding  
134 proteins in Psa strain CFBP7286 genome using FASTA sequence similarity searching tool (EMBL-  
135 EBI, Cambridge, UK). Only identities higher than 60% were considered as acceptable. The

136 corresponding nucleotide sequences in Psa CFBP7286 genome were identified using Geneious  
137 software ver. R8 [34]. Specific qPCR primers were designed using Primer3Plus [35-36].  
138 Thermodynamic properties and secondary structures of the primers and the amplicons were verified  
139 with Beacon Designer™ ver. 8.0 (PREMIER Biosoft, Palo Alto, USA). The list of the primers used  
140 in this study is provided as Online Resource 1. All primer pairs were checked for specificity by end-  
141 point PCR (performed as described for qPCR) using the genomic DNA as the template.

142

#### 143 *Gene expression at different bacterial densities*

144 The expression of genes involved in bacterial motility, biofilm formation, or encoding virulence  
145 effectors or LuxR solos was determined in wild-type Psa strain CFBP7286 cultures grown in LB to  
146 final cell densities of  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$  or  $10^8$  CFU mL<sup>-1</sup>. Three biological replicates were used for  
147 each density.

148 Psa culture volumes containing comparable cell numbers (approx.  $10^6$ ) were sampled for each cell  
149 density. After centrifugation ( $13,000 \times g$ , 4 °C, 10 min), the supernatants were discarded, and the  
150 pellets were stored at -80°C until processing. Total bacterial RNA was extracted using Total RNA  
151 Purification kit (Norgen Biotek Corp., Thorold, CA). RNA purity and quantity were checked using a  
152 NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). An aliquot of 1 µg of  
153 purified RNA was converted to double stranded cDNA by reverse transcription using the High  
154 Capacity cDNA Reverse Transcription kit (Applied Biosystem Life Technologies, Carlsbad, USA)  
155 according to manufacturer's recommendations. The cDNA samples were diluted tenfold, and 3 µL  
156 aliquots of the resulting suspension were used as templates for qPCR, performed with SybrGreen  
157 chemistry (Applied Biosystem Life Technologies, Foster City, USA) in a 96-well spectrofluorometric  
158 thermal cycler StepOnePlus (Thermo Fisher Scientific Inc., Waltham, USA). Each sample was run in  
159 technical triplicate. qPCR cycles were performed as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95  
160 °C for 10 min, 40 cycles at 95 °C for 15 s and 61 °C for 1 min. Melting curve analysis was performed

161 immediately after completion of the qPCR (95 °C for 15 s, 61 °C for 15 s). Target gene expression  
162 was calculated as the relative expression compared to the transcript level of the housekeeping genes  
163 *recA* and *rpoD*, previously adopted for other pseudomonads as stably expressed reference genes [37-  
164 39]. Primer efficiency was assessed using LingRegPCR software [40]. The relative quantification of  
165 gene expression was evaluated through the comparative Ct method [41].

166

#### 167 *Sample treatment with bacterial supernatants, AHLs and 1-undecene*

168 Cell-free supernatants were obtained from Psa cultures grown to the population densities of  $10^5$  (low  
169 density, LD) or  $10^8$  (high density, HD) CFU mL<sup>-1</sup>. For the other species, the supernatants were  
170 obtained from cultures at the end of the log-phase. The cultures were pelleted ( $13,000 \times g$ , 4 °C, 10  
171 min), and the supernatants were sterilised by filtration through a 0.22 µm pore membrane (Millipore,  
172 Billerica, USA).

173 Stock solutions (10 mM) of AHLs, namely C6-OH-, C8-OH-, C10-OH- and C12-OH-homoserine  
174 lactone (HSL) were provided by prof. P. Williams (University of Nottingham, UK). 1-undecene was  
175 purchased from Sigma-Aldrich (St. Louis, USA). Stock 100 mM solutions were made in phosphate  
176 buffer saline.

177

#### 178 *Formation of biofilm*

179 The production of biofilm was evaluated according to Pratt and Kolter [42]. Psa cultures were grown  
180 in LB liquid medium containing AHL (0.01 – 10 µM final concentration), or in cell-free bacterial  
181 supernatants, to a density of  $10^8$  CFU mL<sup>-1</sup>. Psa cultures in fresh, unamended LB medium were used  
182 as the control.

183 A 3-mL aliquot of LB medium was inoculated in a Petri dish (35 mm diameter), at a starting density  
184 of  $10^5$  cells mL<sup>-1</sup>. After inoculation, the capsules were sealed with parafilm and incubated at 27°C

185 with slow shaking (70 rpm). After five days, the plates were thoroughly rinsed with distilled sterile  
186 water and dried for 45 min under laminar hood at room temperature. Then, 3 mL of a crystal violet  
187 water solution (0.5% w/v) (Sigma-Aldrich, St. Louis, USA) were added to each plate. The plates were  
188 incubated for 60 min at room temperature under shaking (70 rpm), and subsequently washed  
189 thoroughly with distilled water, to remove nonspecific staining. For quantitative analysis of biofilms,  
190 crystal violet was re-solubilised by adding 3 ml of ethanol 95%. The absorbance ( $\lambda = 595$  nm) of the  
191 resulting solution was quantified and compared to a blank produced from axenic LB medium.

192

### 193 *Motility phenotype*

194 The occurrence of a swarming phenotype was assessed according to Kinscherf and Willis [43]. LB  
195 plates containing 0.4% agar were amended with AHLs, 1-undecene, or with supernatants obtained  
196 from liquid cultures of Psa (LD and HD) or other bacteria. For each treatment, 1-mL aliquots of  
197 bacterial supernatants, AHLs (0.01 – 10  $\mu$ M in phosphate buffer saline) or 1-undecene (0.5 – 10 mM  
198 in LB) were spread on the plate until complete absorption. LB or phosphate buffer saline were used  
199 as the controls.

200 Subsequently, a sterile filter paper disk (6 mm diameter) was placed on the plate and inoculated with  
201 10  $\mu$ L of a Psa suspension, containing about  $10^7$  CFU mL<sup>-1</sup>. The plate was incubated at 27 °C for 5  
202 days, before observing the bacterial motility phenotype. Each treatment was replicated on 15 to 40  
203 plates.

204

### 205 *Host colonisation*

206 The ability of Psa to colonise kiwifruit plants was tested according to previous work [44], with slight  
207 modifications for the application on *in vitro*-micropropagated plants of *A. chinensis* var. *deliciosa* cv.  
208 Hayward grown in controlled conditions. Psa wild-type or mutant strains were grown in cell-free  
209 bacterial supernatants or in LB medium containing 0.25 or 1  $\mu$ M C6-OH-HSL, to a density of  $10^8$

210 CFU mL<sup>-1</sup>. Psa cultures in unamended LB medium were used as the control. Before inoculation,  
211 bacterial cultures were pelleted by centrifugation (5,000 × g, 4 °C, 5 min) and resuspended in an equal  
212 volume of 10 mM MgSO<sub>4</sub> solution.

213 The plants, grown on MS medium [45] and about 5 cm tall, were inoculated by dipping for 10 s in  
214 the Psa suspension, and kept in a growth chamber for the whole duration of the experiments (22 °C,  
215 70% RH and a light/dark cycle of 16:8 h).

216 Three plants were collected one, three and ten days after inoculation. To determine the populations  
217 of endophytic Psa, the plants were surface-sterilised by successive 1-min washes in 60% ethanol,  
218 15% NaHClO and sterile water, before being frozen in liquid N<sub>2</sub> and stored at -80 °C until molecular  
219 quantification of Psa populations by qPCR [46].

220

#### 221 *Gene expression in response to bacterial supernatants, AHL, and 1-undecene treatments*

222 Gene expression studies were performed on Psa cultures grown to a population density of 10<sup>8</sup> CFU  
223 mL<sup>-1</sup> in bacterial supernatants, or LB amended with AHLs (C6-OH- or C8-OH-HSL, 0.25 or 1 μM)  
224 or 1-undecene (0.5 – 10 mM). Each treatment included three biological replicates, and cultures grown  
225 in LB medium were taken as the negative control.

226 The transcription analysis was carried out as described above, on the same panel of genes considered  
227 for bacterial density effects.

228

#### 229 *Statistical analysis*

230 The statistical significance of differences (assumed for  $p \leq 0.05$ ) was calculated by ANOVA followed  
231 by Tukey's test. The interaction between AHL type and concentration was determined with two-way  
232 ANOVA. The software STATISTICA 7.0 (StatSoft Inc., Tulsa, USA) was used for statistical  
233 elaboration. The significance of differences among percentages was determined according to  
234 Marascuilo's procedure for multiple comparisons among proportions, based on  $\chi^2$  test.

## 235 RESULTS

236

### 237 *Psa* gene expression and phenotypes at different population densities

238 The ability of *Psa* to perceive its own density was first assessed at molecular level through the analysis  
239 of gene expression. To that purpose, several genes were selected based on their described role in cell  
240 density response and/or relevance to social phenotypes, as well as their potential regulation by QS  
241 systems. The selected genes are related to bacterial motility (*fliP*, *rpoN*, *pilA*, *pilC*, *pilO*) or biofilm  
242 formation (*algD*, *wspR*, *wssB*), or encode for virulence effectors (*avrPto1*, *hopD1*, *hopS2*, *hopZ5*) or  
243 quorum sensing-related transcriptional regulators (*psaR1*, *psaR2*, *psaR3*). The analysis revealed that,  
244 at high population densities ( $10^7$ - $10^8$  CFU mL<sup>-1</sup>), several genes, related to biofilm formation (*algD*,  
245 *wspR*, *wssB*), flagellum-mediated motility (*flip* and *rpoN*) and virulence effectors (*hopZ5*, *avrPto1*,  
246 *hopD1* and *hopS2*), resulted up-regulated compared with lower densities (Fig. 1). Based on these  
247 results, population densities of  $10^5$  and  $10^8$  CFU mL<sup>-1</sup>, corresponding respectively to the early and  
248 late logarithmic growth phases (Online Resource 2), were selected for subsequent experiments as  
249 representatives of low (LD) and high bacterial densities (HD), respectively.

250 To investigate whether *Psa* perceived its own density via the recognition of a diffusible signal, the  
251 bacterium was inoculated in cell-free supernatants obtained from *Psa* HD or LD cultures. In both  
252 supernatants, *Psa* growth rate was comparable to that in fresh medium (Online Resource 2). HD  
253 culture supernatant was more effective than supernatant originating from LD cultures in stimulating  
254 both swarming motility (with a three-fold increase of swarming incidence) and biofilm production  
255 (increased by 30% compared to control) (Fig. 2a, 2b). In addition, the endophytic population of *Psa*  
256 in artificially inoculated kiwifruit plants was larger in the early infection stages (i.e. 1 day post-  
257 inoculation, dpi), when the bacterium had been grown in HD supernatant prior to plant inoculation,  
258 suggesting a higher virulence (Fig. 2c). No significant differences in *Psa* population were observed  
259 4-10 dpi.

260 In agreement with phenotypic observation, the expression of genes relevant to motility (*fliP*, *pilA*,  
261 *pilC*, *pilO*) and virulence (*hopZ5*, *hopD1*) showed a significant increase in bacteria grown in HD  
262 culture supernatant (Fig. 2d). Moreover, HD supernatant also stimulated the expression of the LuxR  
263 solo *psaR1*, but not that of *psaR2* and *psaR3*.

264

#### 265 *Responsiveness of Psa to other bacterial species*

266 Cell-free supernatants obtained from cultures (approx.  $10^8$  CFU mL<sup>-1</sup>) of bacteria which share the  
267 same epiphytic niche as Psa (namely, the two kiwifruit plant pathogens, *P. syringae* pv. *syringae* and  
268 *P. viridiflava*, as well as the plant symbiont *P. fluorescens*) were tested for the induction of virulence  
269 effector-related gene expression, motility and biofilm production in Psa cultures.

270 All the tested supernatants promoted both swarming motility and biofilm formation compared to fresh  
271 LB medium (Fig. 3a, 3b). On the other hand, the growth of Psa *in planta* was stimulated only by the  
272 supernatants of kiwifruit pathogens, namely *P. syringae* pv. *syringae* and *P. viridiflava*, in the first  
273 days of infection (from 1 to 3 dpi), whereas no statistical difference was observed at 10 dpi (Fig. 3c).  
274 By contrast, *P. fluorescens* supernatant reduced (although not significantly) Psa growth *in planta*  
275 during the first three days after inoculation.

276 Gene expression analysis, performed on Psa cultures grown in the different cell-free supernatants,  
277 revealed that each of the bacterial supernatants could promote the expression of several genes related  
278 to motility (*rpoN*, *pilC*) and biofilm formation (*algD*, *wssB*, *wspR*), while virulence effector genes  
279 were up-regulated only by *P. syringae* pv. *syringae* or *P. viridiflava* supernatants. Among the putative  
280 receptors of diffusible signals in Psa, only *psaR1* was up-regulated by *P. syringae* pv. *syringae*  
281 supernatant.

282

#### 283 *Involvement of AHLs in Psa interspecies communication*

284 The response of Psa to AHLs added to the growth medium was further evaluated using different  
285 concentrations of pure AHLs (Fig. 4). Concentrations of 1 and 10  $\mu$ M of C6-OH- and C8-OH-HSL  
286 increased the occurrence of swarming motility (Fig. 4a), while the promotion of biofilm formation  
287 was observed at a concentration range of the same AHLs from 0.1 to 0.25  $\mu$ M (Fig. 4b). Unlike tests  
288 in LB medium, in which swarming was observed in 10-20% of control colonies, no swarming was  
289 found in control colonies in presence of PBS alone. Swarming, but not biofilm formation, was also  
290 promoted by C10-OH- and C12-OH-HSL (Online Resource 2). However, since they were less  
291 efficient in inducing Psa social behaviour compared to C6-OH- and C8-OH-HSL, subsequent  
292 experiments concerning endophytic growth and gene expression were carried out with the latter  
293 compounds, at 0.25 and 1  $\mu$ M, the two most effective concentrations in promoting biofilm formation  
294 and motility, respectively. The bacterial growth *in planta* was promoted by C6-OH-HSL (0.25 and 1  
295  $\mu$ M) and C8-OH-HSL (0.25  $\mu$ M) three days post-inoculation (Fig. 4c).

296 Gene expression profiles of Psa cultured in presence of 1  $\mu$ M or 0.25  $\mu$ M of C6-OH- or C8-OH-HSL  
297 fitted well with the observed phenotypes since, in comparison with control, genes related to motility  
298 were down-regulated at low AHL concentration (0.25  $\mu$ M), while those involved in the production of  
299 biofilm and virulence effectors formation were up-regulated (Fig. 5). By contrast, 1  $\mu$ M AHLs  
300 stimulated motility-related genes, whereas the promotion of virulence effectors was non-significant.  
301 In these experiments, concentration effects were significant for all genes but *psaRI*, 2 and 3, while  
302 molecule specificity or combined (molecule  $\times$  concentration) effects were only observed for a few  
303 genes (Online resource 2).

304 Since Psa responds to AHL treatment, if produced by bacterial strains sharing the same epiphytic  
305 niche, these compounds could participate in interspecies communication between Psa and  
306 neighbouring bacteria. AHL production by the three selected *Pseudomonas* strains living on the same  
307 host plant was assessed through bioassays performed using the well-known *C. violaceum* strain  
308 CV026 and *A. tumefaciens* strain NT1 (pZLR4) [33]. Both bioassays were positive with *P. syringae*

309 *pv. syringae*, in line with previous works reporting the production of AHLs by several strains of Pss  
310 [47,48], whereas only the *A. tumefaciens* NT1 (pZLR4) assay was positive in presence of *P.*  
311 *fluorescens*, confirming the likely production of only long-chain AHLs by this species [49]. Finally,  
312 both assays confirmed *P. viridiflava* as a non AHL-producer, as previously reported [48].

313

#### 314 *Role of Psa luxR solos in bacterial signalling and AHL perception*

315 The role of LuxR solos in Psa responses to bacterial supernatants and AHLs was examined using the  
316 knock-out mutants for the *psaR1*, *psaR2* or *psaR3* genes (named Psa-mR1, 2 and 3), previously  
317 described [27]. Lower levels of *hopZ5* transcription were observed in *psaR2* and *psaR3* mutants  
318 grown in LB medium, compared to the WT and *psaR1* mutant strains (Fig. 6).

319 Biofilm formation and motility were assessed in wild-type Psa and Psa-mR1, 2 and 3 strains, grown  
320 in LD or HD Psa supernatants or supernatants from bacterial epiphytes (Fig. 7). Psa-mR1 swarming  
321 motility was significantly promoted with respect to wild type after growth in *P. syringae pv. syringae*  
322 and *P. fluorescens* supernatants, while biofilm production was reduced with the same treatments.  
323 Contrariwise, Psa-mR3 swarming motility was significantly reduced by *P. fluorescens* supernatant,  
324 and biofilm formation increased in *P. syringae pv. syringae* and *P. fluorescens* supernatants.  
325 Swarming was also less frequent in the three LuxR solos mutants the wild-type strain when grown in  
326 LB (control) or in HD supernatant.

327 Biofilm formation, motility and plant colonisation by those mutants were assessed also in presence  
328 of different AHL concentrations. In presence of 1  $\mu$ M AHL, swarming motility was more intensely  
329 induced in the *psaR1* knock-out strain than in the wild type, and it was abolished in the *psaR3*-  
330 defective strain (Fig. 8a). On the other hand, in presence of 0.25  $\mu$ M C6-OH- and C8-OH-HSL,  
331 biofilm production was reduced in the *psaR1* knock-out mutant and enhanced in the *psaR3* knock-  
332 out mutant (Fig. 8b). Mutating *psaR2* had no effect on AHL-mediated biofilm and motility phenotype.  
333 Plant colonisation was dramatically reduced in *psaR2*- and *psaR3*-defective strains, and unaffected in

334 *psaRI* knock-out mutants. The addition of 0.25  $\mu$ M C6-OH-HSL recovered Psa-mR3, but not Psa-  
335 mR2 virulence (Fig. 8c).

336

337 *Responsiveness of Psa to 1-undecene*

338 The growth and swarming motility of Psa was tested after treatment with 1-undecene. The lowest  
339 concentration inducing measurable effects was 1 mM. In such conditions, swarming and *in planta*  
340 growth were significantly reduced (Fig. 9).

## 341 DISCUSSION

342

### 343 *Perception of density-related molecule(s) regulating QS-mediated phenotypes by Psa*

344 The ability of bacterial cells to perceive the population density of their own species, known as quorum  
345 sensing (QS), has been described in numerous pseudomonads grown in artificial media, including for  
346 instance the human pathogen *Pseudomonas aeruginosa* and the phytopathogenic *P. syringae* pv.  
347 *syringae* [50]. The availability of molecular and phenotypical data obtained mainly with such model  
348 microorganisms was exploited to study Psa responsiveness to environmental signals and to  
349 investigate some ecological relationships possibly involved in the epiphytic survival and the early  
350 stages of plant colonisation by Psa. To adapt to the growing conditions, it is expected that Psa can  
351 perceive multiple classes of compounds and adjust its own metabolism as a direct (e.g. for nutrient  
352 compounds or metabolic by-products) or indirect (e.g. through signalling cascades) consequence.

353 In this study, we show that, at high cell density, the expression of genes related to biofilm formation  
354 and cell motility is induced in Psa. These processes are commonly observed as QS-related responses  
355 induced by high cell density in other bacterial species [51,52]. Interestingly, the growth of Psa in  
356 filter-sterilised supernatants obtained from HD cultures stimulated the expression of phenotypes  
357 (increased swarming and endophytic colonisation) observed in HD cultures. The similarity of growth  
358 curves of Psa in the different supernatants demonstrates that the observed responses are probably not  
359 due to limiting nutrient conditions, even in the supernatants of late log-phase bacterial cultures  
360 (Online Resource 2). Overall, these results thus clearly point out that Psa, when reaching a certain  
361 population density, produces diffusible QS signals involved in intra-species communication.

362 Psa population density did not show a linear correlation with gene expression levels. In particular,  
363 the expression of genes involved in biofilm formation (i.e. *algD*, *wspR*) reached a maximum at a  
364 bacterial concentration of  $10^7$  CFU mL<sup>-1</sup> and then decreased drastically at  $10^8$  CFU mL<sup>-1</sup>. This may  
365 be related to QS-regulated biofilm disassembly, as previously observed [53]. In contrast, genes related

366 to motility (*fliP*, *rpoN*) and virulence (*hopZ5*, *avrPto1*, *hopD1*, *hopS2*) are up-regulated at higher  
367 bacterial densities, in a similar way at both  $10^7$  and  $10^8$  CFU mL<sup>-1</sup>. Although these traits all depend  
368 on population density, their biological significance may differ: swarming promotes bacterial spread  
369 and exploration, whereas biofilm formation allows a more efficient exploitation of resources by  
370 metabolic specialisation of the cells in different positions of the colony, and endophytic colonisation  
371 grants access to a source of nutrients precluded to other microbial competitors.

372 According to quorum sensing definition, a critical population threshold (identified for Psa at approx.  
373  $10^7$  CFU mL<sup>-1</sup>) represents the switch for the activation of genes underlying high density-related  
374 phenotypes. It may be speculated that, besides the production and perception of QS specific signal(s)  
375 reaching a concentration threshold at high cell density, multiple QS-related signalling pathways may  
376 coexist in Psa, integrating other signals (such as nutrient availability, environmental stresses and  
377 competition) to elicit the most appropriate response.

378

#### 379 *Psa responsiveness to diffusible signal molecule(s) produced by neighbouring bacteria*

380 Due to the limited chemical variability of bacterial signals and the partial sensitivity of receptors to  
381 non-cognate signals, an inter-species communication among epiphytic bacteria was predicted to  
382 occur. Indeed, for instance, it was observed that *P. syringae* pv. *syringae* responded to up to 7% of  
383 culturable epiphytic bacteria collected from random plants [51]. However, all of them belonged to  
384 the genera *Pseudomonas*, *Erwinia* or *Pantoea*, confirming that cross-communication is most frequent  
385 in taxonomically and/or environmentally associated bacterial groups. Accordingly, the cell-free  
386 supernatants from two bacterial pathogens sharing the same host plant than Psa, namely *P. syringae*  
387 pv. *syringae* and *P. viridiflava*, promote Psa biofilm formation, motility and growth within the host  
388 plant, suggesting that, during epiphytic growth and the initial phases of host colonisation, they may  
389 act as a pathogenic consortium. Indeed, growing evidence highlighted that pathogens often do not  
390 operate independently, but their virulence in natural conditions is expressed after the formation of a

391 synergistic consortium with other pathogens, thus promoting disease incidence and development  
392 [54,55].

393 Cross-talk among plant-associated bacteria was observed in previous researches, which proposed that  
394 one possible function may be to benefit signal-emitting species by influencing the behaviour of its  
395 neighbours [51]. This view implies that the stability of microbial communities is the result of the co-  
396 selection of bacterial species or strains, which respond to QS signals in a coordinate manner and  
397 consistently. Such conditions may be more frequent in communities co-evolved on a specific host  
398 plant, leading for instance to the formation of a pathogen consortium [52; this work]. On the other  
399 hand, epiphytic bacteria, not specifically associated to *P. syringae* pv. *syringae*, reduced the virulence  
400 of the latter by means of QS signals [51]. Thus, a further clarification of the signal exchange between  
401 Psa and other *Actinidia spp.*-associated bacteria may help pointing out communication mechanisms,  
402 which may be exploited for the control of kiwifruit bacterial canker.

403

#### 404 *Putative signal molecules for Psa QS-mediated phenotype regulation*

405 Since it has been reported that Psa does not produce AHLs [27] but swarming motility, biofilm  
406 production and *in planta* growth were all influenced by Psa HD supernatant, other compounds  
407 occurring in bacterial species must be involved in Psa bacterial cell-cell communication. Among the  
408 putative candidate signals, the volatile compound 1-undecene was shown to be produced by several  
409 *Pseudomonas* species and pathovars, including Psa [28,29,56]. Although no clear function was  
410 attributed to this molecule [31,32], it has been previously reported that bacterial volatile compounds  
411 (such as acetic acid, indole, 2-amino-acetophenone) may play a role as a signal for cell-cell  
412 communication and QS [57-59]. In this study, a similar role may be played by 1-undecene, which  
413 induces the expression of biofilm-regulating diguanylate cyclases (*wssB* gene). Such effect was  
414 observed at a concentration of 1 mM 1-undecene, possibly much higher than the actual release in Psa  
415 liquid cultures. Nevertheless, because of its high solubility in apolar environments, 1-undecene

416 concentration in bacterial biofilm matrices might reach locally higher levels, possibly similar to the  
417 ones tested in our experiments. Alternatively, since 1-undecene treatment reduces Psa growth, the  
418 observed effects may reflect its toxicity at the tested concentrations, rather than a physiological  
419 function. Further experiments will thus be required to define more precisely the possible function of  
420 that compound in Psa.

421 In canonical Gram-negative bacteria QS system, AHLs represent the key signal molecules for  
422 phenotype regulation. Interestingly, they are involved in both intra- and interspecific bacterial  
423 communication, allowing bacteria to detect the presence of other species to adapt their behaviour [51,  
424 60]. Although Psa does not produce AHLs itself, it was proposed that it may sense AHLs produced  
425 by neighbouring bacteria [27]. Accordingly, the treatment of Psa with different AHLs led to QS-  
426 related phenotype induction. A certain chemical specificity was observed, based on the length of the  
427 acyl moiety, with only short-chain AHLs such as C6-OH- and C8-OH-HSL, but not C10-OH- and  
428 C12-OH-HSL, eliciting biofilm formation (Online Resource 2). Interestingly, *P. syringae* pv.  
429 *syringae* produces such short-chain AHLs [51; this work]. Psa responsiveness to AHLs may thus be  
430 biologically relevant for its interaction with *Pss* for regulating its behaviour within the host plant. In  
431 addition, different phenotypes were induced by different concentrations of the same AHLs: low AHLs  
432 concentrations promoted biofilm formation, while high AHLs concentrations stimulated Psa  
433 swarming motility. Thus, both the chemical nature of the signal as well as its concentration are  
434 relevant for the elicitation of a specific response.

435 Overall, the existence of several integrated signals, as well as AHL perception specificities (between  
436 short- and long-chain AHLs) and concentration gradients likely play a role in determining the degree  
437 of association between Psa and other microbial species of the phyllosphere. Moreover, AHL-based  
438 signal may be further integrated with other diffusible molecules, produced by *P. fluorescens* (only  
439 long chain AHLs producer) and *P. viridiflava* (non AHL producer). Such signal network, likely

440 organized in a hierarchical manner [13], may be required for the regulation of Psa behaviour within  
441 the host plant.

442

#### 443 *Function of Psa LuxR solos in QS-related responses*

444 The expression of Psa *luxR* homologues (namely *psaR1*, *psaR2*, *psaR3*) did not correlate with Psa  
445 population density increase. Since a common feature of LuxR transcriptional regulators is to regulate  
446 their own expression upon activation by the population density-indicating auto-inducer molecule  
447 [12], this suggests that Psa does not produce PsaR1/R2/R3 cognate signal compounds.

448 The existence of a system for AHL recognition, but not production, in Psa led to postulate an adaptive  
449 eavesdropping role for LuxR solos of Psa, as suggested for other species [19]. We showed here that  
450 *psaR1* gene expression was promoted by both exogenously applied AHLs and the culture supernatant  
451 of *P. syringae* pv. *syringae* that produces short-chain AHLs. These data suggest that PsaR1 may bind  
452 AHLs produced by neighbouring bacteria to mediate QS-related responses, or, in alternative, PsaR1  
453 induction may take place downstream of a signalling cascade induced by another AHL receptor,  
454 according to the complex hierarchical interconnectivity regulating several LuxR sensors, as reported  
455 for the Las and Rhl systems in *Pseudomonas aeruginosa* [49].

456 Regarding the putative function of LuxR-like sensors in Psa, *psaR1*- and *psaR3*-defective mutants  
457 showed an opposite behaviour when treated with AHLs, indicating an opposite function of the two  
458 LuxR solos in regulating these processes [27]: PsaR1 inhibits swarming and promotes biofilm  
459 formation, while PsaR3 is required for swarming and negatively regulates biofilm formation.  
460 Although recognising the same compounds, these LuxR solos probably trigger different signal  
461 cascades, further confirming that multiple levels of integration and regulation exist between signal  
462 perception and phenotype expression, concurring to its fine tuning. On the other hand, mutating *psaR2*  
463 dramatically reduced endophytic growth, but had no effect on AHL-mediated biofilm and motility

464 phenotype, suggesting that PsaR2 does not participate in AHL signal perception, in line with its  
465 putative role in interkingdom communication [27].

466

#### 467 *Conclusions*

468 The study of bacterial phenotype differentiation and microbial synergism may be an underestimated  
469 aspect of plant pathology, because of the theoretical and technical difficulties associated with such  
470 studies [55]. For instance, the social phenotypes considered in this work may contribute to virulence  
471 only under particular conditions, and other genes may compensate or regulate the function of the  
472 selected ones. In this regard, a transcriptomic analysis of Psa interactions with the environment,  
473 including other bacterial residents of the phyllosphere, may provide a deeper and more  
474 comprehensive understanding of Psa ecology.

475 However, crucial information on the mechanisms of epiphytic colonisation and infection can be  
476 obtained when focusing on the epiphytic biocoenosis, rather than on a single bacterial species. The  
477 elucidation of such mechanisms in Psa might lead to the use of new biochemical and/or  
478 microbiological tools for the control of the bacterial canker of kiwifruit by interfering with the  
479 pathogen perception of its ecological contour.

480

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486

487 **CONFLICT OF INTEREST**

488 The authors declare that they have no conflict of interest.

489

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657

658 FIGURE CAPTIONS

659

660 **Fig. 1**

661 Relative expression of genes involved in bacterial motility, biofilm formation, production of virulence  
662 effectors and signal perception in *Pseudomonas syringae* pv. *actinidiae* cultured in liquid Luria-  
663 Bertani medium at cell densities ranging from  $10^4$  to  $10^8$  CFU mL<sup>-1</sup>. Data are indicated as mean  $\pm$   
664 standard error (n = 3). For each gene, different letters indicate a statistically significant ( $p < 0.05$ )  
665 difference in expression levels, calculated by ANOVA and Tukey's test

666

667 **Fig. 2**

668 Effects of supernatants of *Pseudomonas syringae* pv. *actinidiae* cultures in liquid Luria-Bertani  
669 medium at low ( $10^5$  CFU mL<sup>-1</sup>) (white bars) and high ( $10^8$  CFU mL<sup>-1</sup>) (grey bars) cell densities on  
670 cultures of the same bacterium: (a) percentage of colonies showing a swarming motility phenotype;  
671 (b) estimation of biofilm production; (c) bacterial endophytic growth in *in vitro* *Actinidia chinensis*  
672 var. *deliciosa* plants; (d) expression of a panel of genes related to bacterial motility, biofilm  
673 formation, production of virulence effectors and signal perception, expressed as relative amount of  
674 transcript compared to the housekeeping genes *recA* and *rpoD*. In panels **b – d**, data are shown as  
675 mean  $\pm$  standard error (n = 3). Different letters indicate significant differences among treatments  
676 according to Marascuilo's procedure (a) or to ANOVA followed by Tukey's test (b – d)

677

678 **Fig. 3**

679 Effects of supernatants of cultures of *P. syringae* pv. *syringae* (Pss), *P. viridiflava* (Pv) and *P.*  
680 *fluorescens* (Pf) in liquid Luria-Bertani on *P. syringae* pv. *actinidiae*: (a) percentage of colonies  
681 showing a swarming motility phenotype; (b) estimation of biofilm production; (c) bacterial  
682 endophytic growth in *in vitro* *Actinidia chinensis* var. *deliciosa* plants; (d) expression of a panel of

683 genes related to bacterial motility, biofilm formation, production of virulence effectors and signal  
684 perception, expressed as relative amount of transcript compared to the housekeeping genes *recA* and  
685 *rpoD*. In panels **b – d**, data are shown as mean  $\pm$  standard error (n = 3). Different letters indicate  
686 significant differences among treatments according to Marascuilo's procedure (**a**) or to ANOVA  
687 followed by Tukey's test (**b – d**)

688

689 **Fig. 4**

690 Effect of acyl-homoserine lactones on motility, biofilm formation and virulence of *Pseudomonas*  
691 *syringae* pv. *actinidiae* (Psa): (**a**) percentage of bacterial cultures showing swarming motility after  
692 treatment with a 0.1 – 10  $\mu$ M C6-OH- or C8-OH-homoserine lactone (HSL) solution in phosphate  
693 buffer saline; (**b**) production of biofilm after treatment with 0.1 – 10  $\mu$ M C6-OH- or C8-OH-HSL;  
694 (**c**) endophytic growth of Psa in *in vitro* *Actinidia chinensis* var. *deliciosa* plants after treatment with  
695 0.25  $\mu$ M (left) or 1  $\mu$ M (right) C6-OH- or C8-OH-HSL. Different lower-case (C6-OH-HSL) or upper-  
696 case (C8-OH-HSL) letters indicate significant differences among different concentrations of the same  
697 compound. In panels **b – c**, data are shown as mean  $\pm$  standard error (n = 3). In panel (**c**), different  
698 letters indicate significant differences at the same time point. Statistical significance was determined  
699 by Marascuilo's procedure (**a**) or ANOVA followed by Tukey's test (**b, c**)

700

701 **Fig. 5**

702 Expression of genes related to motility, biofilm formation, production of virulence effectors and  
703 signal perception in *Pseudomonas syringae* pv. *actinidiae* cultures in liquid Luria-Bertani medium,  
704 amended with 0.25  $\mu$ M or 1  $\mu$ M C6-OH- (**a**) or C8-OH-HSL (**b**). Data (mean  $\pm$  standard error, n = 3)  
705 are expressed as the relative amount of transcript compared to the housekeeping genes *recA* and *rpoD*.

706 For each gene, different letters indicate significant differences among treatments according to two-  
707 way ANOVA followed by Tukey's test

708

709 **Fig. 6**

710 Expression of genes related to motility, biofilm formation, production of virulence effectors and  
711 signal perception in wild-type *Pseudomonas syringae* pv. *actinidiae* and knock-out mutants for *luxR*  
712 solo genes, grown in liquid Luria-Bertani medium to a  $10^8$  CFU mL<sup>-1</sup> population density. Data ( mean  
713  $\pm$  standard error, n = 3) are expressed as the relative amount of transcript compared to the  
714 housekeeping genes *recA* and *rpoD*. Different letters indicate significant differences among  
715 treatments, according to ANOVA followed by Tukey's test

716

717 **Fig. 7**

718 Effect of cell-free supernatants deriving from *P. syringae* pv. *syringae*, *P. viridiflava*, *P. fluorescens*  
719 and *Pseudomonas syringae* pv. *actinidiae* (Psa; LD = low density, HD = high density) on swarming  
720 motility (**a**) and biofilm formation (**b**) of wild-type Psa and knock-out mutants for *luxR* solo genes.  
721 For each treatment, different letters indicate significant differences among mutants, according to  
722 Marascuilo's test (**a**) or ANOVA followed by Tukey's test (**b**)

723

724 **Fig. 8**

725 Effect of acyl-homoserine lactones on motility, biofilm formation and virulence of *Pseudomonas*  
726 *syringae* pv. *actinidiae* (Psa) knock-out mutants for *luxR* solo genes: (**a**) percentage of bacterial  
727 cultures showing swarming motility after treatment with 1  $\mu$ M C6-OH- or C8-OH-homoserine  
728 lactone (HSL) solutions in phosphate buffer saline; (**b**) production of biofilm after treatment with

729 0.25  $\mu$ M C6-OH- or C8-OH-HSL; (c) endophytic growth in *in vitro* *Actinidia chinensis* var. *deliciosa*  
730 plants of Psa wild-type and mutant strains grown in LB (left) or LB amended with 0.25  $\mu$ M C6-OH-  
731 HSL (right). In panels b – c, data are shown as mean  $\pm$  standard error (n = 3). Different letters indicate  
732 significant differences among mutants within each treatment and/or time point, according to  
733 Marascuilo’s test (a) or ANOVA followed by Tukey’s test (b, c)

734

735 **Fig. 9**

736 Effects of 1-undecene (0.5 or 1 mM) on cultures of *Pseudomonas syringae* pv. *actinidiae* in liquid  
737 Luria-Bertani medium: (a) percentage of colonies showing a swarming motility phenotype; (b)  
738 bacterial growth kinetics in liquid cultures; (c) expression of a panel of genes related to bacterial  
739 motility, biofilm formation, production of virulence effectors and signal perception. In panels b – c,  
740 data are shown as mean  $\pm$  standard error (n = 3). Different letters indicate significant differences  
741 among treatments, according to Marascuilo’s procedure (a) or ANOVA followed by Tukey’s test (b,  
742 c)

743

744 [ONLINE RESOURCES](#)

745

746 **Online Resource 1**

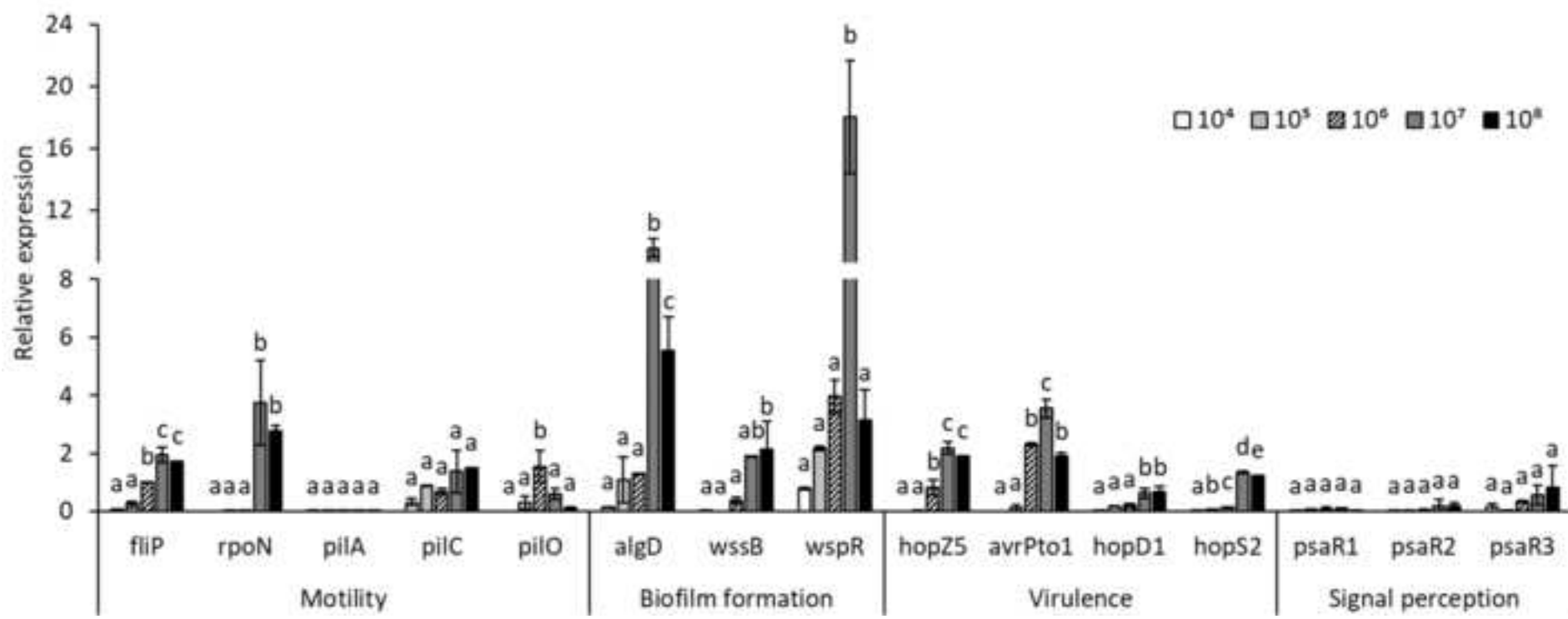
747 List of *Pseudomonas syringae* pv. *actinidiae* genes putatively responding to cell density and/or  
748 implied in social behaviour

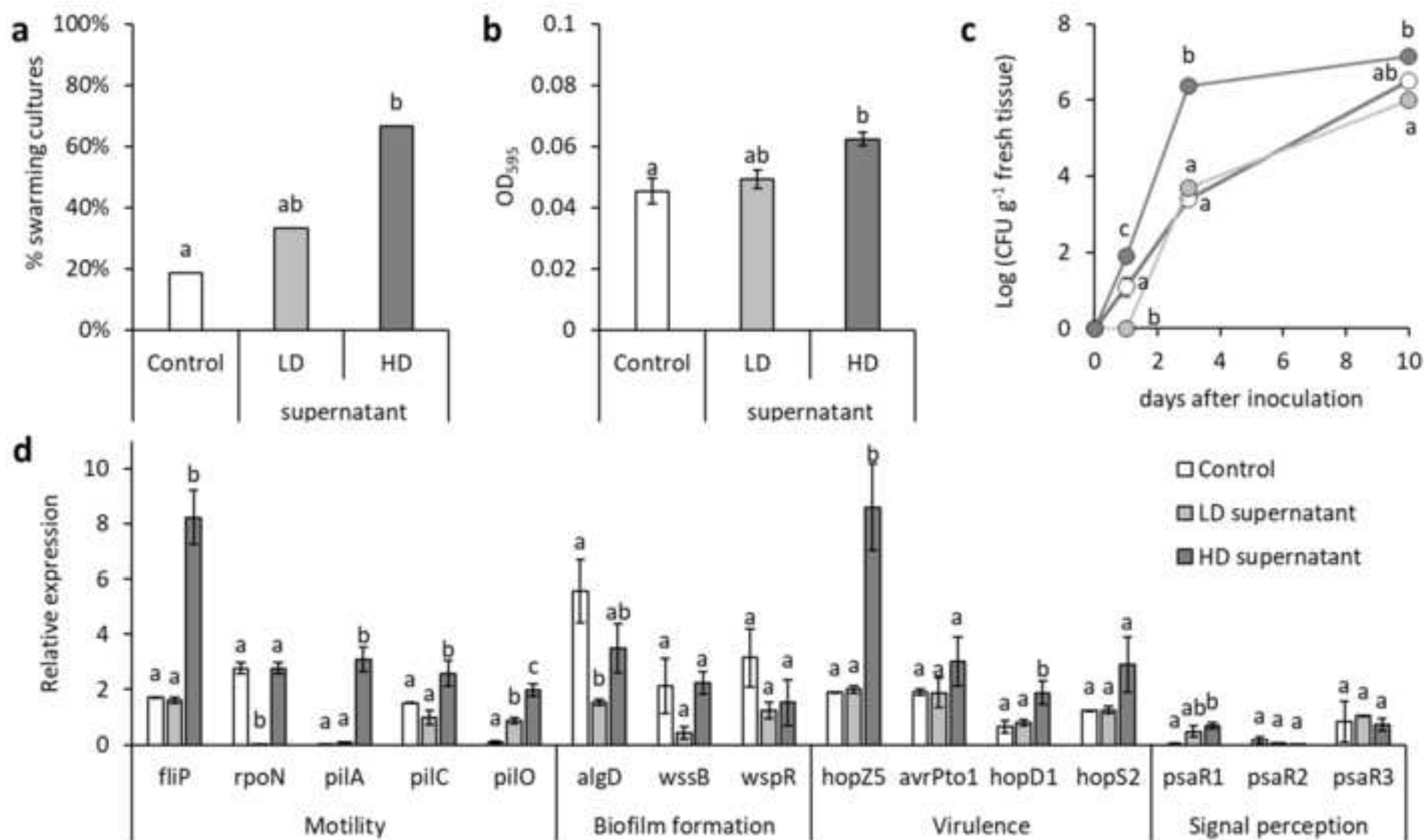
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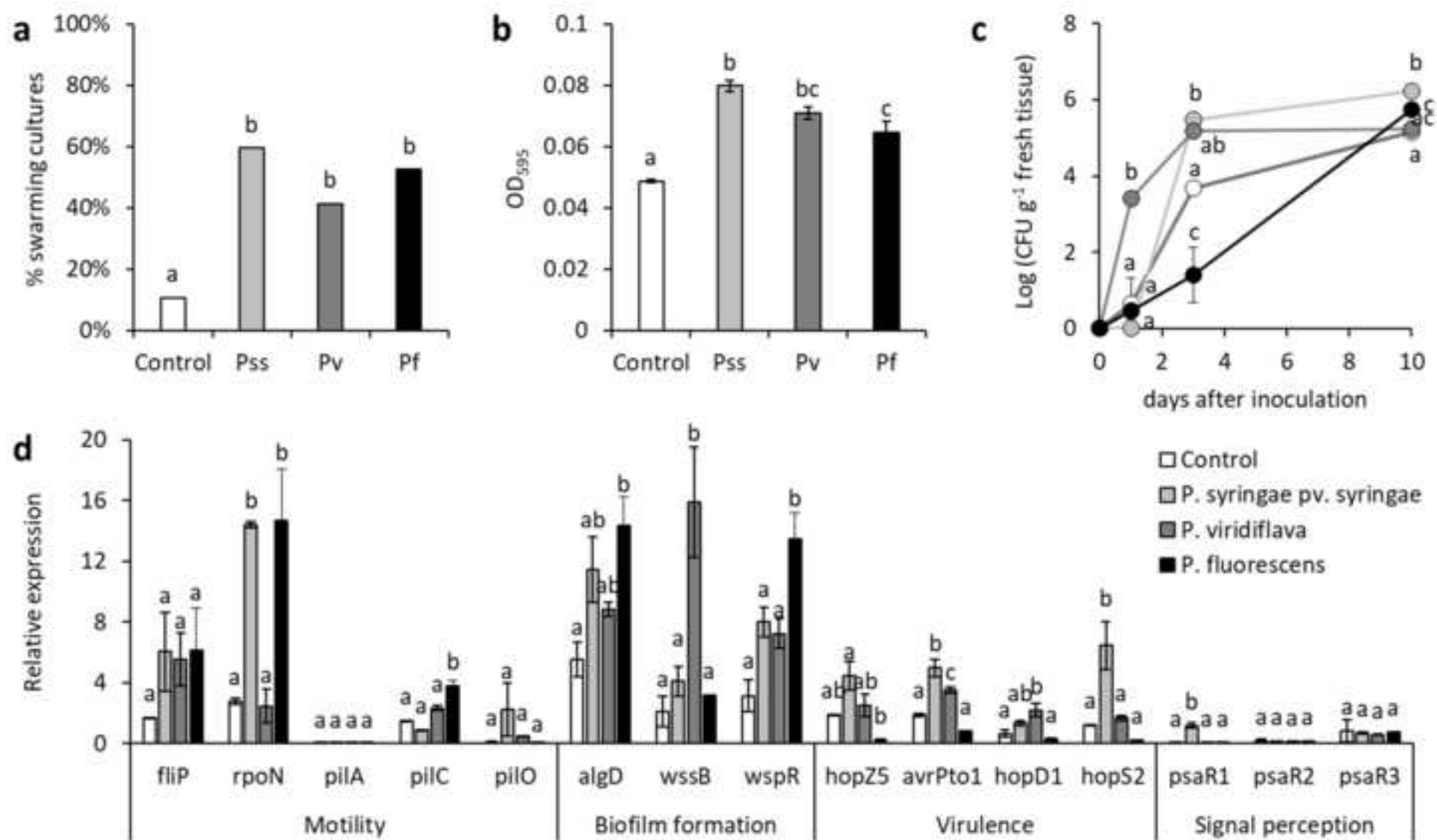
750 **Online Resource 2**

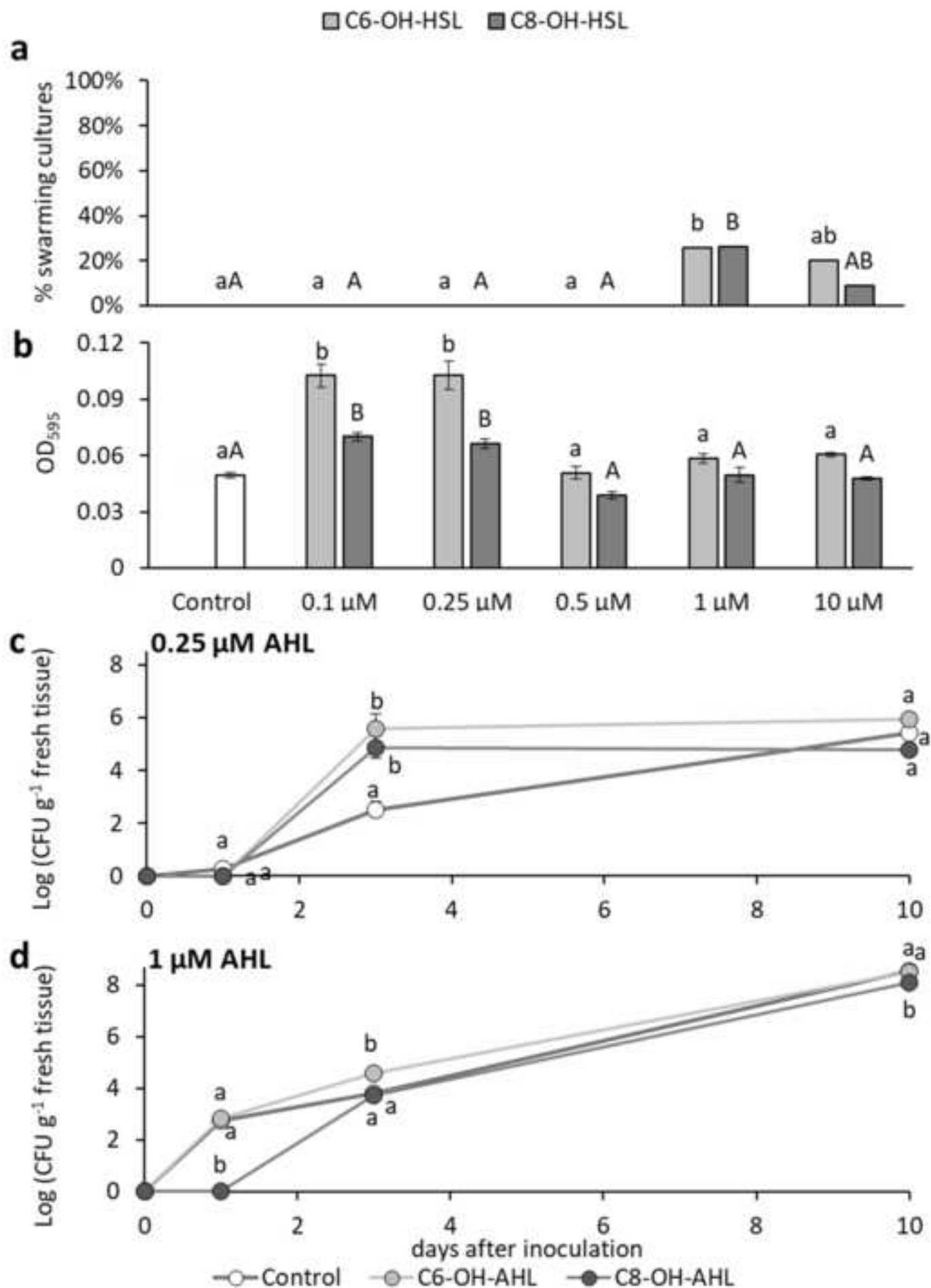
751 **(a)** Growth of *Pseudomonas syringae* pv. *actinidiae* (Psa) in cell-free supernatants of *P. putida* strain  
752 IBE3, *P. syringae* pv. *syringae*, *P. viridiflava* and *P. fluorescens*. **(b)** Growth of Psa in cell-free  
753 supernatants of low density (LD) or high density (HD) Psa cultures. **(c)** percentage of Psa cultures  
754 showing swarming motility after treatment with 1  $\mu$ M C6-OH-, C8-OH-, C10-OH- or C12-OH-  
755 homoserine lactone (HSL) solutions in phosphate buffer saline. **(d)** production of biofilm by Psa after  
756 treatment with 0.25  $\mu$ M C6-OH-, C8-OH-, C10-OH- or C12-OH-HSL. **(e)** expression of genes related  
757 to biosurfactant production in wild-type Psa grown in LD or HD culture supernatants, and in  
758 presence/absence of 1  $\mu$ M C6-OH-HSL, indicated as relative amount of transcript compared to the  
759 housekeeping genes *recA* and *rpoD*. ANOVA followed by Tukey's test (**a, b, d, e**) or Marascuilo's  
760 test (**c**) were performed. Different letters indicate significant differences. **(f)** table of *p* values obtained  
761 by two-way ANOVA on gene expression data presented in fig. 5, considering concentration (0, 0.25  
762 or 1  $\mu$ M) and molecule (C6-OH- or C8-OH-HSL) as the factors. Significant effects ( $p \leq 0.05$ ) are  
763 highlighted in bold

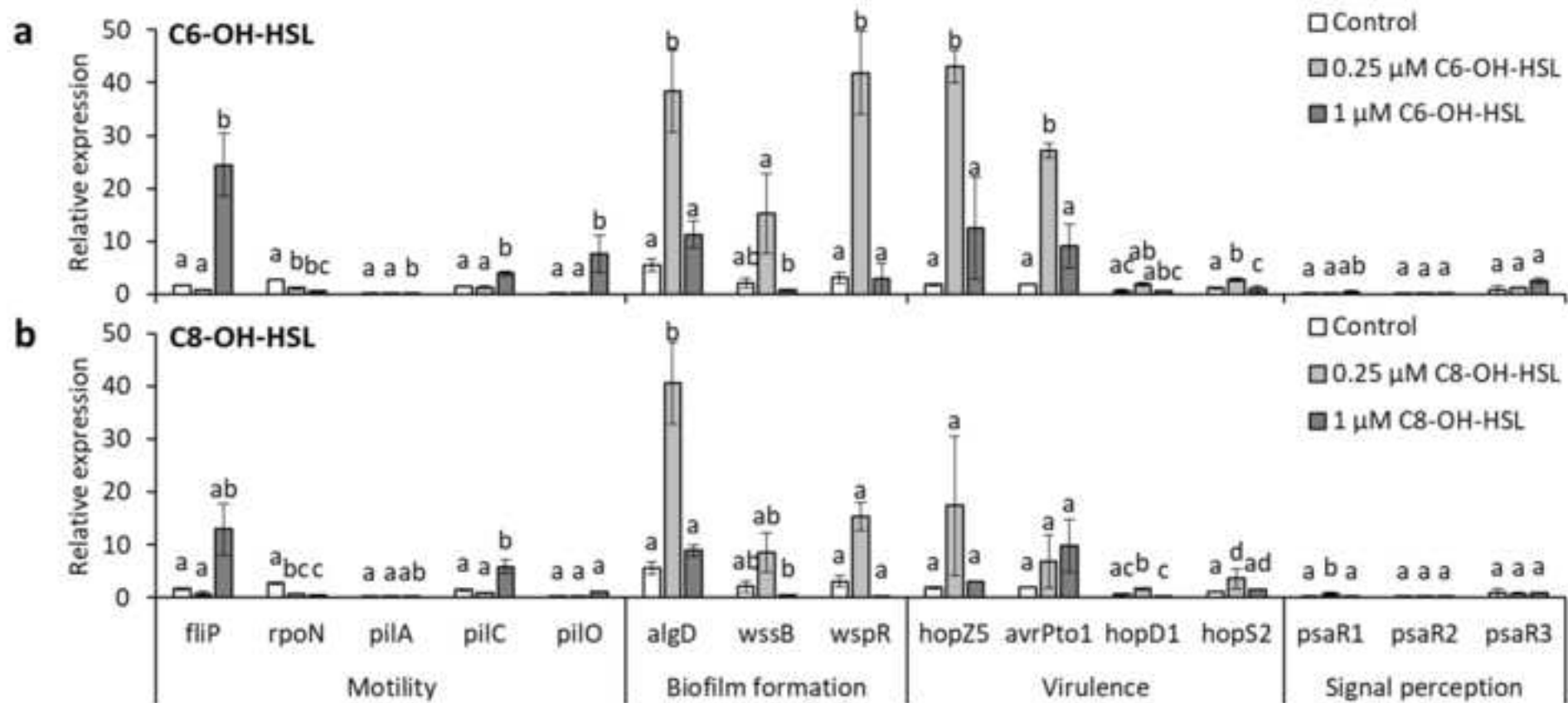
Figure 1

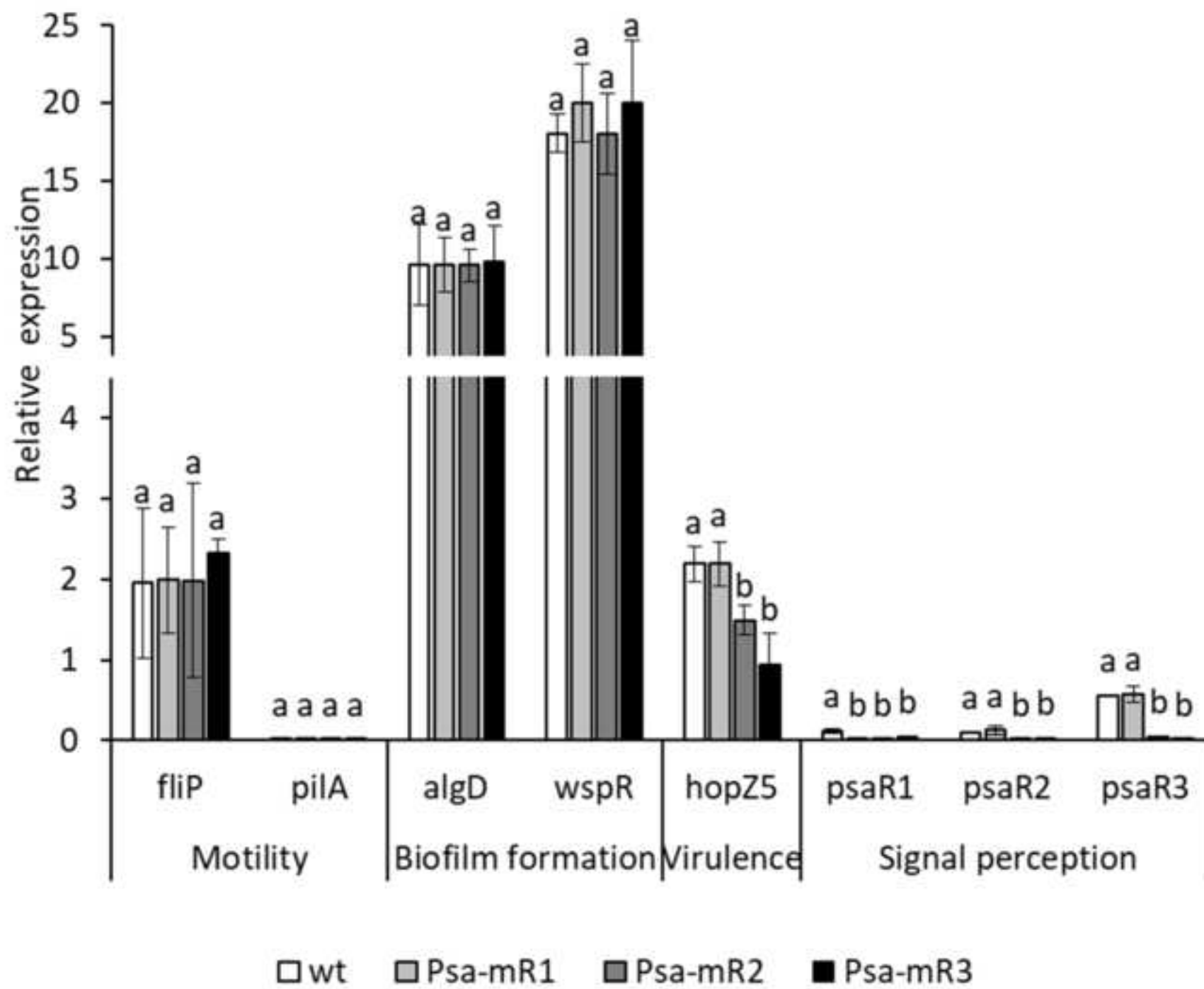


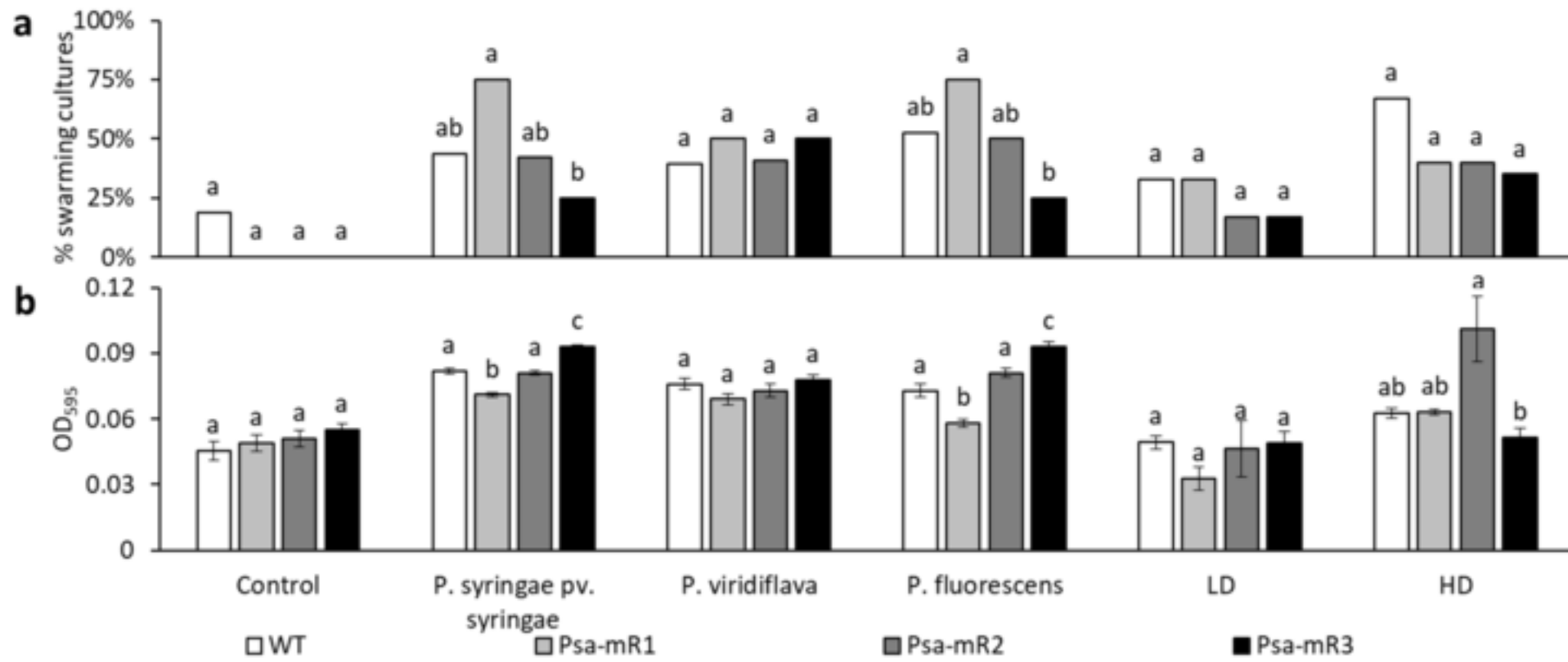


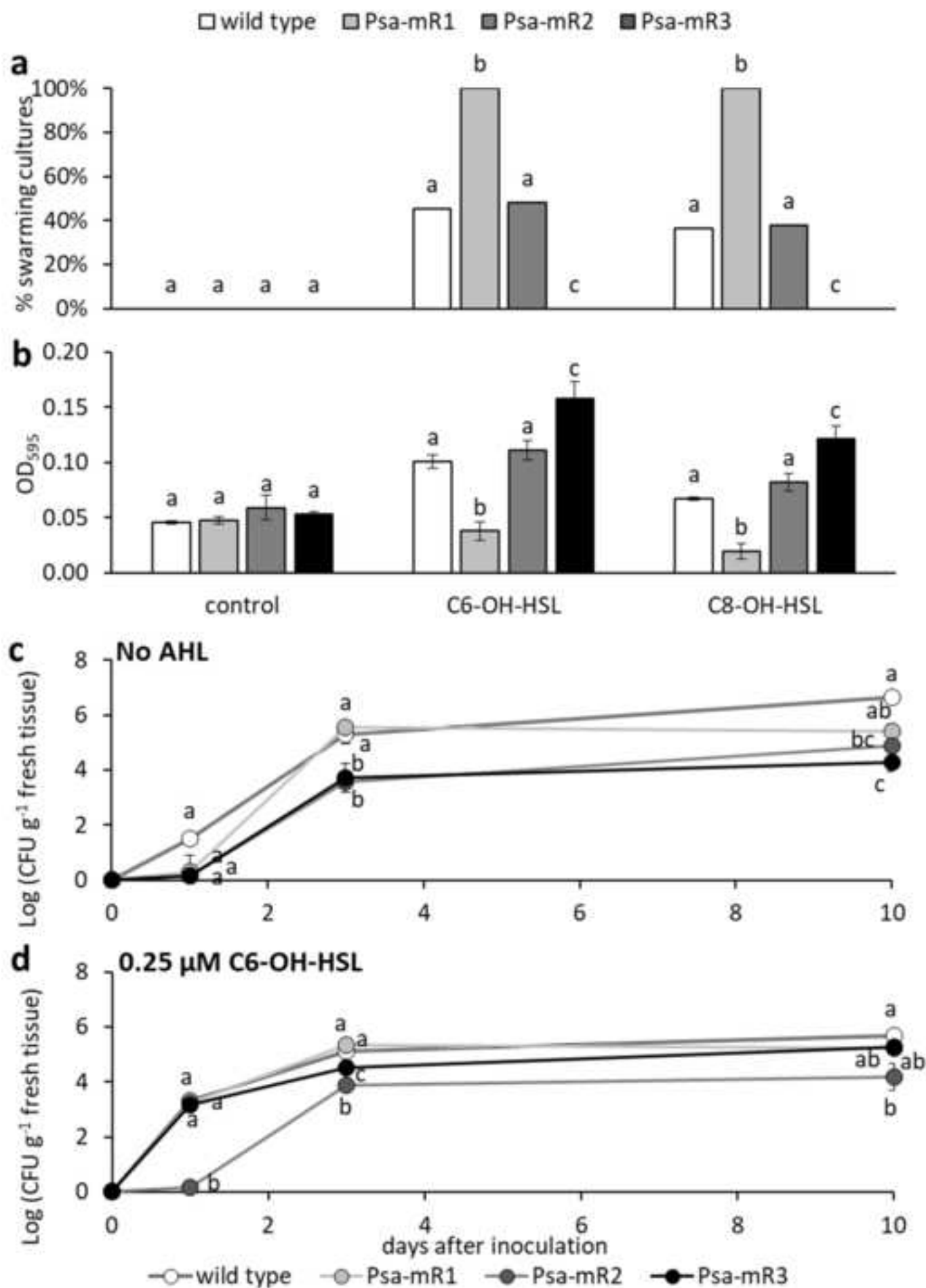


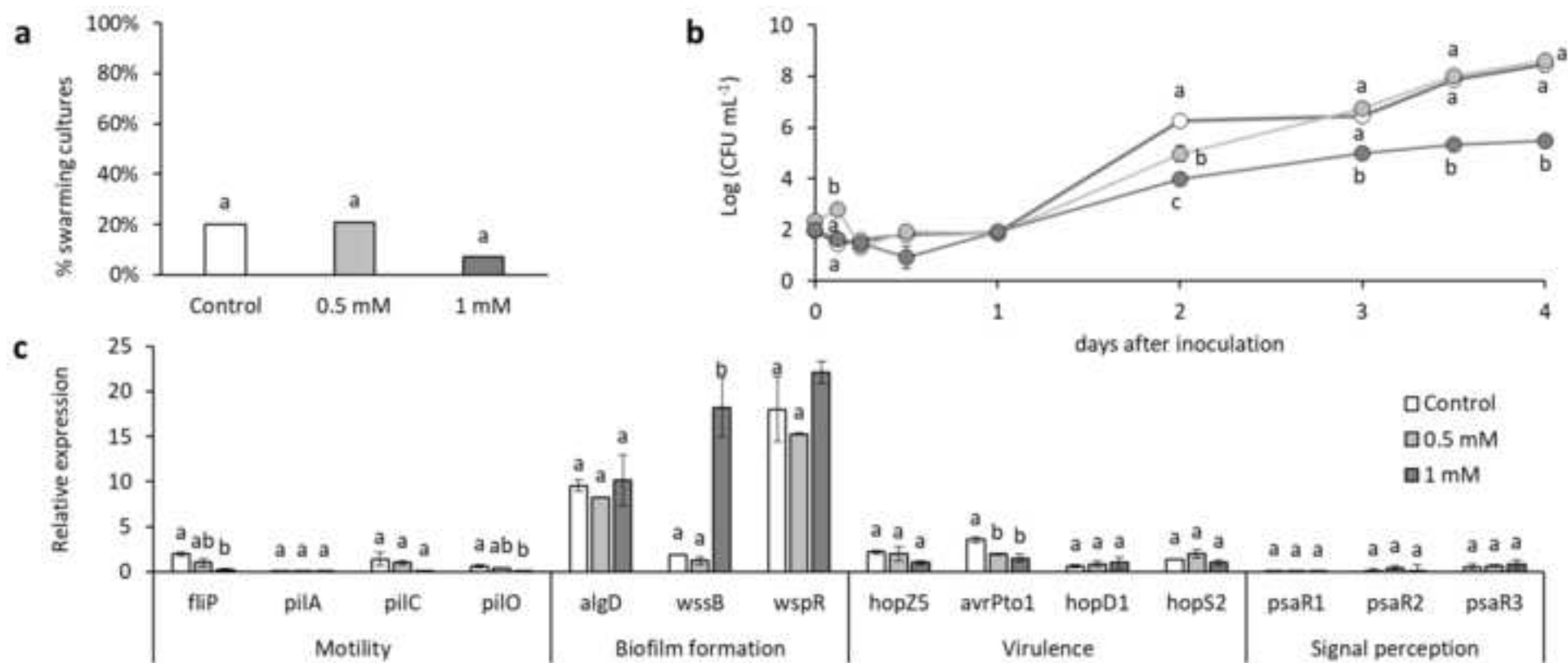


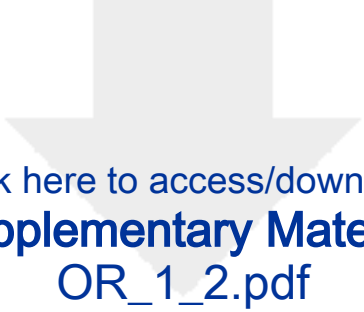












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