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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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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABSTRACT

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

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

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

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

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

INTRODUCTION

The phyllosphere is a complex ecosystem, in which the host plant provides the primary source of nutrients (exudates, cell wall derivatives) supporting the survival of the epiphytic microflora. However, the phyllosphere is an inhospitable, oligotrophic and competitive environment, where microorganisms must adapt to sudden and drastic changes in environmental conditions and to limited and scattered resources [1]. In these harsh conditions, the competition among microbial species could be very high and lead to mutual exclusion [2,3]. On the other hand, the formation of symbiotic consortia, for instance through the reciprocal exchange of metabolites, is another strategy to overcome these limitations [4-6]. Thus, epiphytic microorganisms form complex communities, including plant symbionts, commensals, pathogens and opportunists, where the action of each individual species strictly depends on the network of ecological interactions inside and between each microbial population [3]. For instance, a pathogen might express its virulence only when the microbial composition of the surrounding communities is favourable [7,8]. The study of ecological relationships within multispecies communities has therefore become an emerging issue in plant-microbe interaction and plant pathology. One of the main factors coordinating the dynamics within microbial communities is the production, perception and response to signals among bacterial cells of a same species or belonging to different species. Several microbial communication systems, both intra- and interspecies, mediated by signals of different chemical natures, have been discovered and studied so far [9]. In Gram-negative proteobacteria, N-acyl homoserine lactones (AHLs) represent the most common signals mediating quorum sensing (QS) responses, i.e. the regulation of bacterial behaviour through modulation of gene expression in response to population density [10,11]. Signal specificity of AHLs is determined by the nature of their acyl moiety, i.e. the length of its carbon chain and the substitution at position C3. The archetypical AHL-QS system is based on a LuxI-LuxR protein pair, where the LuxI synthase is responsible for AHL biosynthesis and the LuxR receptor acts as a transcriptional regulator upon AHL binding [12]. LuxI/LuxR-encoding genes are usually adjacently located, forming operons in bacterial genomes. Several LuxI/LuxR pairs can also co-exist in a single genome, leading to a real hierarchical QS network for bacterial behaviour control [13]. However, additional QS-type LuxR homologues have been identified, which lack a cognate LuxI, and were thus termed LuxR 'solos' [14,15]. These receptors, largely present in bacteria, might respond to AHLs released by other species in the environment [15], other diffusible componds, such as pyrones [16], biosurfactants [17] and volatile compounds [18], or even eukaryotic compounds, including plant signal molecules in the case of plant-associated bacteria [19]. In the latter case, LuxR solos would not play a role in QS *per se* but rather in interkingdom communication between bacteria and their host plant.

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

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

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

The release of volatile compounds by Psa and their biological effects on kiwifruit plants have been investigated previously [28]. In contrast, Psa responses to airborne signals are less known. The semivolatile 1-undecene is one of the main compounds released by Psa and several other *Pseudomonas* species [29,30], but no signalling function has been described for it [31,32].

This work examined the social behaviour and some virulence traits of Psa in response to the microflora present in *Actinidia* phyllosphere. The induction of bacterial motility and biofilm formation, which both contribute to epiphytic colonisation, was observed *in vitro*, together with the regulation of the expression of genes involved in these processes. Moreover, the effect on Psa fitness was also evaluated *in planta*, in terms of bacterial growth under controlled conditions. The experiments aimed at determining (i) the ability of Psa to perceive its own population density or (ii) the presence of other epiphytic bacteria; (iii) the role of AHLs and 1-undecene in mediating bacterial communication; (iv) the role of Psa LuxR solos in the perception of bacterial signals.

MATERIALS AND METHODS

Bacterial species, culture conditions and bacteria quantification

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

Population densities during logarithmic bacterial growth in liquid cultures were determined by measuring their optical density at λ = 600 nm (OD_{600}) and confirmed by counting the number of colony forming units (CFU), after plating 10-fold serial dilutions of the bacterial culture on LB-agar medium. A standard curve of correspondence between OD_{600} and population density assessed by plate counts was produced for each strain prior to experiments.

Selection of target genes

Genes were selected based on their potential regulation through QS mechanisms, putative role in cell density response and/or relevance to social phenotypes: bacterial motility (*fliP*, *pilA*, *pilC*, *pilO*), biofilm formation (*algD*, *wspR*, *wssB*), virulence effectors (*avrPto1*, *hopD1*, *hopS2*, *hopZ5*), biosurfactant production (*rhlA*, *syfA*) and quorum sensing (*psaR1*, *2*, *3*) (Supporting information Table S1). A TBLASTN search was performed with the amino acid sequences of the corresponding proteins in Psa strain CFBP7286 genome using FASTA sequence similarity searching tool (EMBL-EBI, Cambridge, UK). Only identities higher than 60% were considered as acceptable. The corresponding nucleotide sequences in Psa CFBP7286 genome were identified using Geneious software ver. R8 [34]. Specific qPCR primers were designed using Primer3Plus [35-36]. Thermodynamic properties and secondary structures of the primers and the amplicons were verified with Beacon Designer™ ver. 8.0 (PREMIER Biosoft, Palo Alto, USA). The list of the primers used in this study is provided as Online Resource 1. All primer pairs were checked for specificity by end-point PCR (performed as described for qPCR) using the genomic DNA as the template.

Gene expression at different bacterial densities

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

Psa culture volumes containing comparable cell numbers (approx. 10^6) were sampled for each cell density. After centrifugation (13,000 × *g*, 4 °C, 10 min), the supernatants were discarded, and the pellets were stored at -80°C until processing. Total bacterial RNA was extracted using Total RNA Purification kit (Norgen Biotek Corp., Thorold, CA). RNA purity and quantity were checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). An aliquot of 1 µg of purified RNA was converted to double stranded cDNA by reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem Life Technologies, Carlsbad, USA) according to manufacturer's recommendations. The cDNA samples were diluted tenfold, and 3 µL aliquots of the resulting suspension were used as templates for qPCR, performed with SybrGreen chemistry (Applied Biosystem Life Technologies, Foster City, USA) in a 96-well spectrofluorometric thermal cycler StepOnePlus (Thermo Fisher Scientific Inc., Waltham, USA). Each sample was run in technical triplicate. qPCR cycles were performed as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 61 °C for 1 min. Melting curve analysis was performed immediately after completion of the qPCR (95 °C for 15 s, 61 °C for 15 s). Target gene expression was calculated as the relative expression compared to the transcript level of the housekeeping genes *recA* and *rpoD*, previously adopted for other pseudomonads as stably expressed reference genes [37-39]. Primer efficiency was assessed using LingRegPCR software [40]. The relative quantification of gene expression was evaluated through the comparative Ct method [41].

Sample treatment with bacterial supernatants, AHLs and 1-undecene

Cell-free supernatants were obtained from Psa cultures grown to the population densities of 10^5 (low density, LD) or 10^8 (high density, HD) CFU mL^{-1}. For the other species, the supernatants were obtained from cultures at the end of the log-phase. The cultures were pelleted (13,000 × *g*, 4 °C, 10 min), and the supernatants were sterilised by filtration through a 0.22 μm pore membrane (Millipore, Billerica, USA).

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

Formation of biofilm

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

A 3-mL aliquot of LB medium was inoculated in a Petri dish (35 mm diameter), at a starting density of 10^5 cells mL^{-1}. After inoculation, the capsules were sealed with parafilm and incubated at 27°C with slow shaking (70 rpm). After five days, the plates were thoroughly rinsed with distilled sterile water and dried for 45 min under laminar hood at room temperature. Then, 3 mL of a crystal violet water solution (0.5% w/v) (Sigma-Aldrich, St. Louis, USA) were added to each plate. The plates were incubated for 60 min at room temperature under shaking (70 rpm), and subsequently washed thoroughly with distilled water, to remove nonspecific staining. For quantitative analysis of biofilms, crystal violet was re-solubilised by adding 3 ml of ethanol 95%. The absorbance ($\lambda = 595$ nm) of the resulting solution was quantified and compared to a blank produced from axenic LB medium.

Motility phenotype

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

Subsequently, a sterile filter paper disk (6 mm diameter) was placed on the plate and inoculated with 10 μL of a Psa suspension, containing about 10^7 CFU mL^{-1}. The plate was incubated at 27 °C for 5 days, before observing the bacterial motility phenotype. Each treatment was replicated on 15 to 40 plates.

Host colonisation

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

CFU mL^{-1}. Psa cultures in unamended LB medium were used as the control. Before inoculation, bacterial cultures were pelleted by centrifugation (5,000 × *g*, 4 °C, 5 min) and resuspended in an equal volume of 10 mM $MgSO_4$ solution.

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

Three plants were collected one, three and ten days after inoculation. To determine the populations of endophytic Psa, the plants were surface-sterilised by successive 1-min washes in 60% ethanol, 15% NaHClO and sterile water, before being frozen in liquid N_2 and stored at -80 °C until molecular quantification of Psa populations by qPCR [46].

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

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

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

Statistical analysis

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

RESULTS

Psa gene expression and phenotypes at different population densities

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

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

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

Responsiveness of Psa to other bacterial species

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

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

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

Involvement of AHLs in Psa interspecies communication

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

Gene expression profiles of Psa cultured in presence of 1 μM or 0.25 μM of C6-OH- or C8-OH-HSL fitted well with the observed phenotypes since, in comparison with control, genes related to motility were down-regulated at low AHL concentration (0.25 μM), while those involved in the production of biofilm and virulence effectors formation were up-regulated (Fig. 5). By contrast, 1 μM AHLs stimulated motility-related genes, whereas the promotion of virulence effectors was non-significant. In these experiments, concentration effects were significant for all genes but *psaR1*, *2* and *3*, while molecule specificity or combined (molecule × concentration) effects were only observed for a few genes (Online resource 2).

Since Psa responds to AHL treatment, if produced by bacterial strains sharing the same epiphytic niche, these compounds could participate in interspecies communication between Psa and neighbouring bacteria. AHL production by the three selected *Pseudomonas* strains living on the same host plant was assessed through bioassays performed using the well-known *C. violaceum* strain CV026 and *A. tumefaciens* strain NT1 (pZLR4) [33]. Both bioassays were positive with *P. syringae* pv. *syringae*, in line with previous works reporting the production of AHLs by several strains of Pss [47,48], whereas only the *A. tumefaciens* NT1 (pZLR4) assay was positive in presence of *P. fluorescens*, confirming the likely production of only long-chain AHLs by this species [49]. Finally, both assays confirmed *P. viridiflava* as a non AHL-producer, as previously reported [48].

Role of Psa luxR solos in bacterial signalling and AHL perception

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

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

Biofilm formation, motility and plant colonisation by those mutants were assessed also in presence of different AHL concentrations. In presence of 1 μM AHL, swarming motility was more intensely induced in the *psaR1* knock-out strain than in the wild type, and it was abolished in the *psaR3*-defective strain (Fig. 8a). On the other hand, in presence of 0.25 μM C6-OH- and C8-OH-HSL, biofilm production was reduced in the *psaR1* knock-out mutant and enhanced in the *psaR3* knock-out mutant (Fig. 8b). Mutating *psaR2* had no effect on AHL-mediated biofilm and motility phenotype. Plant colonisation was dramatically reduced in *psaR2*- and *psaR3*-defective strains, and unaffected in *psaR1* knock-out mutants. The addition of 0.25 µM C6-OH-HSL recovered Psa-mR3, but not Psa-mR2 virulence (Fig. 8c).

Responsiveness of Psa to 1-undecene

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

DISCUSSION

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

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

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

Psa population density did not show a linear correlation with gene expression levels. In particular, the expression of genes involved in biofilm formation (i.e. *algD*, *wspR*) reached a maximum at a bacterial concentration of 10^7 CFU mL^{-1} and then decreased drastically at 10^8 CFU mL^{-1}. This may be related to QS-regulated biofilm disassembly, as previously observed [53]. In contrast, genes related to motility (*fliP*, *rpoN*) and virulence (*hopZ5*, *avrPto1*, *hopD1*, *hopS2*) are up-regulated at higher bacterial densities, in a similar way at both 10^7 and 10^8 CFU mL^{-1}. Although these traits all depend on population density, their biological significance may differ: swarming promotes bacterial spread and exploration, whereas biofilm formation allows a more efficient exploitation of resources by metabolic specialisation of the cells in different positions of the colony, and endophytic colonisation grants access to a source of nutrients precluded to other microbial competitors.

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

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

Due to the limited chemical variability of bacterial signals and the partial sensitivity of receptors to non-cognate signals, an inter-species communication among epiphytic bacteria was predicted to occur. Indeed, for instance, it was observed that *P. syringae* pv. *syringae* responded to up to 7% of culturable epiphytic bacteria collected from random plants [51]. However, all of them belonged to the genera *Pseudomonas*, *Erwinia* or *Pantoea*, confirming that cross-communication is most frequent in taxonomically and/or environmentally associated bacterial groups. Accordingly, the cell-free supernatants from two bacterial pathogens sharing the same host plant than Psa, namely *P. syringae* pv. *syringae* and *P. viridiflava*, promote Psa biofilm formation, motility and growth within the host plant, suggesting that, during epiphytic growth and the initial phases of host colonisation, they may act as a pathogenic consortium. Indeed, growing evidence highlighted that pathogens often do not operate independently, but their virulence in natural conditions is expressed after the formation of a synergistic consortium with other pathogens, thus promoting disease incidence and development [54,55].

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

Putative signal molecules for Psa QS-mediated phenotype regulation

Since it has been reported that Psa does not produce AHLs [27] but swarming motility, biofilm production and *in planta* growth were all influenced by Psa HD supernatant, other compounds occurring in bacterial species must be involved in Psa bacterial cell-cell communication. Among the putative candidate signals, the volatile compound 1-undecene was shown to be produced by several *Pseudomonas* species and pathovars, including Psa [28,29,56]. Although no clear function was attributed to this molecule [31,32], it has been previously reported that bacterial volatile compounds (such as acetic acid, indole, 2-amino-acetophenone) may play a role as a signal for cell-cell communication and QS [57-59]. In this study, a similar role may be played by 1-undecene, which induces the expression of biofilm-regulating diguanylate cyclases (*wssB* gene). Such effect was observed at a concentration of 1 mM 1-undecene, possibly much higher than the actual release in Psa liquid cultures. Nevertheless, because of its high solubility in apolar environments, 1-undecene concentration in bacterial biofilm matrices might reach locally higher levels, possibly similar to the ones tested in our experiments. Alternatively, since 1-undecene treatment reduces Psa growth, the observed effects may reflect its toxicity at the tested concentrations, rather than a physiological function. Further experiments will thus be required to define more precisely the possible function of that compound in Psa.

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

Overall, the existence of several integrated signals, as well as AHL perception specificities (between short- and long-chain AHLs) and concentration gradients likely play a role in determining the degree of association between Psa and other microbial species of the phyllosphere. Moreover, AHL-based signal may be further integrated with other diffusible molecules, produced by *P. fluorescens* (only long chain AHLs producer) and *P. viridiflava* (non AHL producer). Such signal network, likely organized in a hierarchical manner [13], may be required for the regulation of Psa behaviour within the host plant.

Function of Psa LuxR solos in QS-related responses

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

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

Regarding the putative function of LuxR-like sensors in Psa, *psaR1*- and *psaR3*-defective mutants showed an opposite behaviour when treated with AHLs, indicating an opposite function of the two LuxR solos in regulating these processes [27]: PsaR1 inhibits swarming and promotes biofilm formation, while PsaR3 is required for swarming and negatively regulates biofilm formation. Although recognising the same compounds, these LuxR solos probably trigger different signal cascades, further confirming that multiple levels of integration and regulation exist between signal perception and phenotype expression, concurring to its fine tuning. On the other hand, mutating *psaR2* dramatically reduced endophytic growth, but had no effect on AHL-mediated biofilm and motility phenotype, suggesting that PsaR2 does not participate in AHL signal perception, in line with its putative role in interkingdom communication [27].

Conclusions

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

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

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE CAPTIONS

Fig. 1

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

Fig. 2

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

Fig. 3

Effects of supernatants of cultures of *P. syringae* pv. *syringae* (Pss), *P. viridiflava* (Pv) and *P. fluorescens* (Pf) in liquid Luria-Bertani on *P. syringae* pv. *actinidiae*: (**a**) percentage of colonies showing a swarming motility phenotype; (**b**) estimation of biofilm production; (**c**) bacterial endophytic growth in *in vitro Actinidia chinensis* var. *deliciosa* plants; (**d**) expression of a panel of genes related to bacterial motility, biofilm formation, production of virulence effectors and signal perception, expressed as relative amount of transcript compared to the housekeeping genes *recA* and *rpoD*. In panels **b** – **d**, data are shown as mean ± standard error (n = 3). Different letters indicate significant differences among treatments according to Marascuilo's procedure (**a**) or to ANOVA followed by Tukey's test (**b** – **d**)

Fig. 4

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

Fig. 5

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

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

Fig. 6

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

Fig. 7

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

Fig. 8

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

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

Fig. 9

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

ONLINE RESOURCES

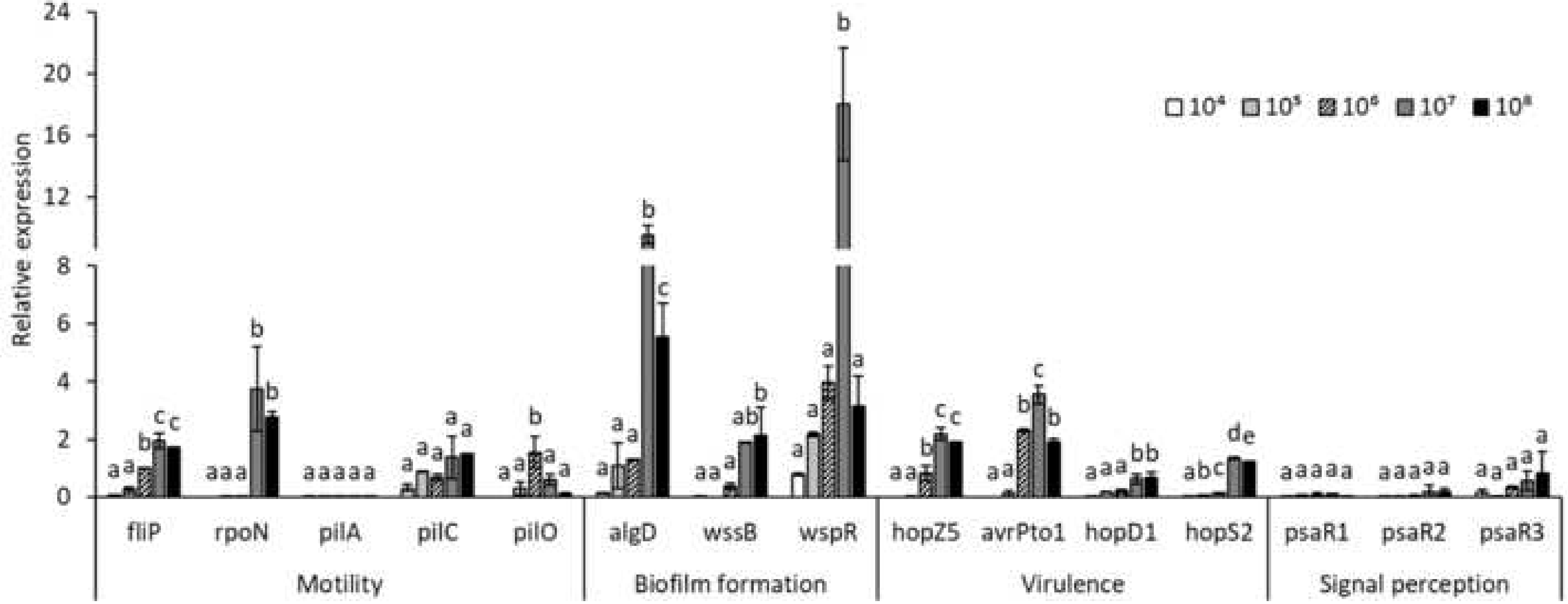
Online Resource 1

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

Online Resource 2

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

Figure 1


Relative expression
0
2
4
6
8
12
16
20
24
10^4
10^5
10^6
10^7
10^8
fliP
rpoN
pilA
pilC
pilO
algD
wssB
wspR
hopZ5
avrPto1
hopD1
hopS2
psaR1
psaR2
psaR3
Motility
Biofilm formation
Virulence
Signal perception
a a b c c
a a a b b
a a a a a
a a a a a
a a b a a
a a a b c
a a a ab b
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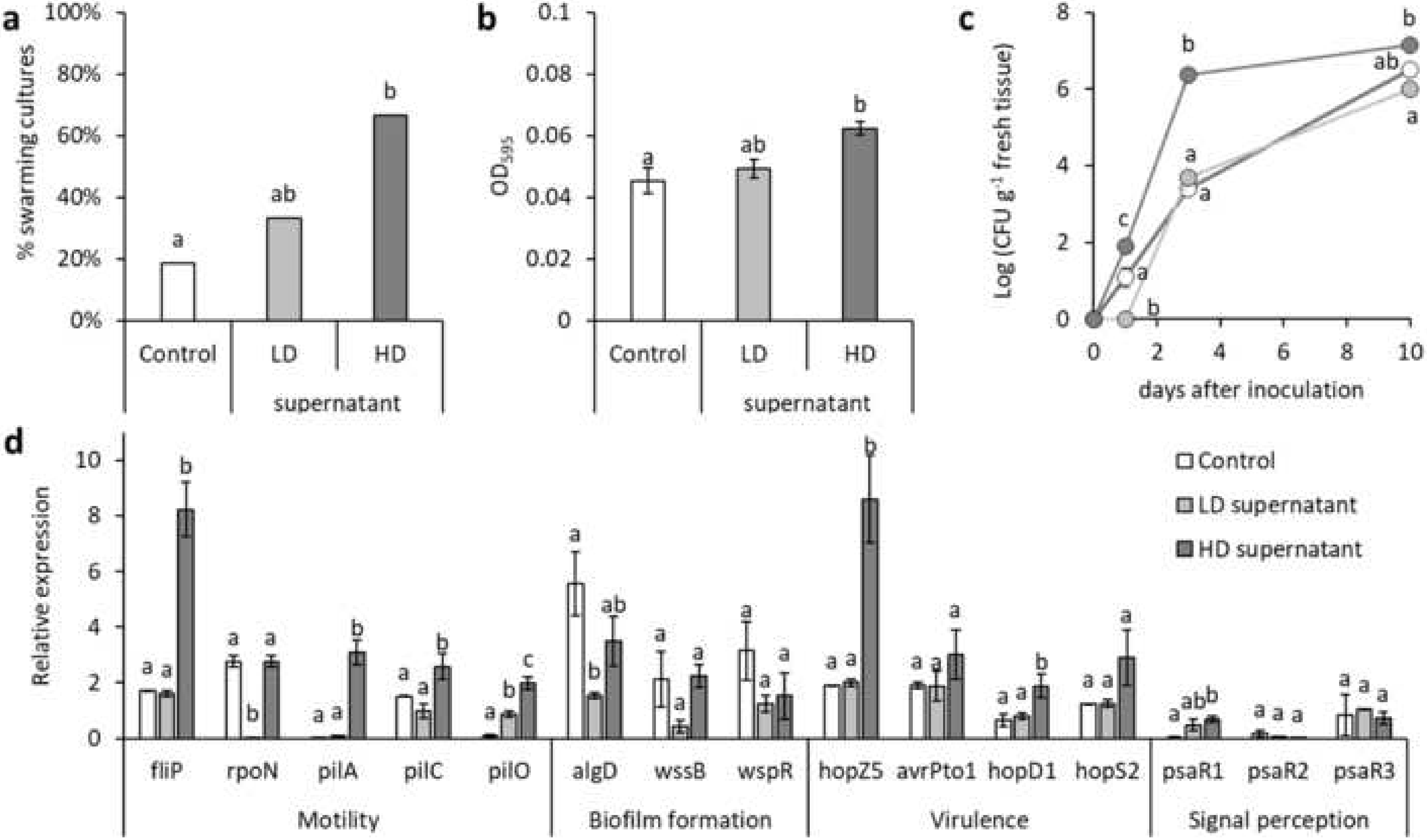
a
% swarming cultures
100%
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ab
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Control
LD
HD
supernatant
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OD_{595}
0.1
0.08
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a
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LD
HD
supernatant
c
Log (CFU g^{-1} fresh tissue)
8
6
4
2
0
b
b
ab
a
a
c
a
a
b
0
2
4
6
8
10
days after inoculation
d
Relative expression
10
8
6
4
2
0
□ Control
▨ LD supernatant
■ HD supernatant
fliP
rpoN
pilA
pilC
pilO
algD
wssB
wspR
hopZ5
avrPto1
hopD1
hopS2
psaR1
psaR2
psaR3
Motility
Biofilm formation
Virulence
Signal perception

Figure 3

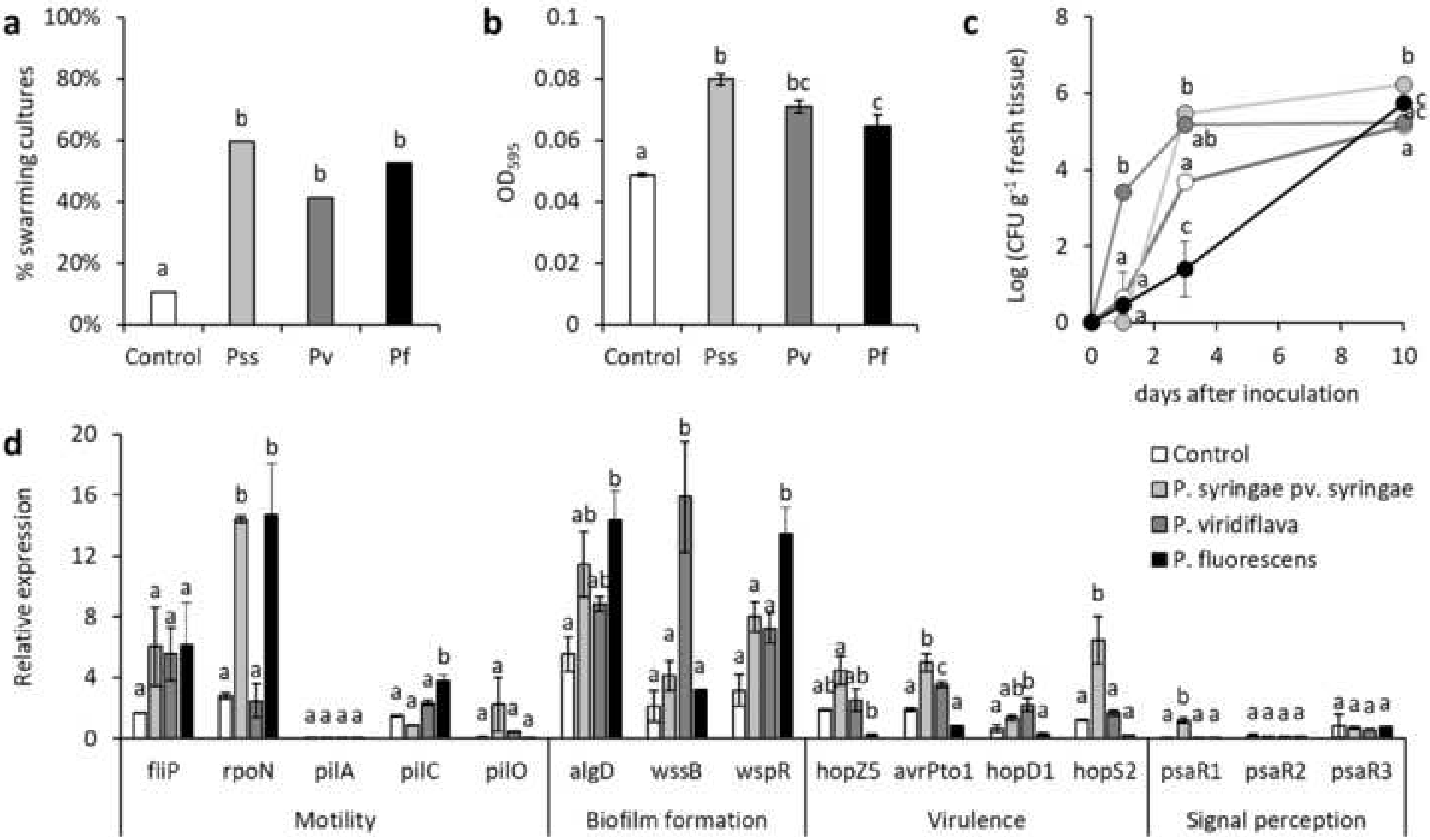

a
% swarming cultures
100%
80%
60%
40%
20%
0%
Control
Pss
Pv
Pf
a
b
b
b
b
OD_{595}
0.1
0.08
0.06
0.04
0.02
0
Control
Pss
Pv
Pf
a
b
bc
c
c
Log (CFU g^{-1} fresh tissue)
8
6
4
2
0
0
2
4
6
8
10
days after inoculation
b
b
ab
a
a
a
c
a
b
c
ac
a
d
Relative expression
20
16
12
8
4
0
fliP
rpoN
pilA
pilC
pilO
algD
wssB
wspR
hopZ5
avrPto1
hopD1
hopS2
psaR1
psaR2
psaR3
Motility
Biofilm formation
Virulence
Signal perception
☐ Control
▨ P. syringae pv. syringae
▨ P. viridiflava
■ P. fluorescens

Figure 4

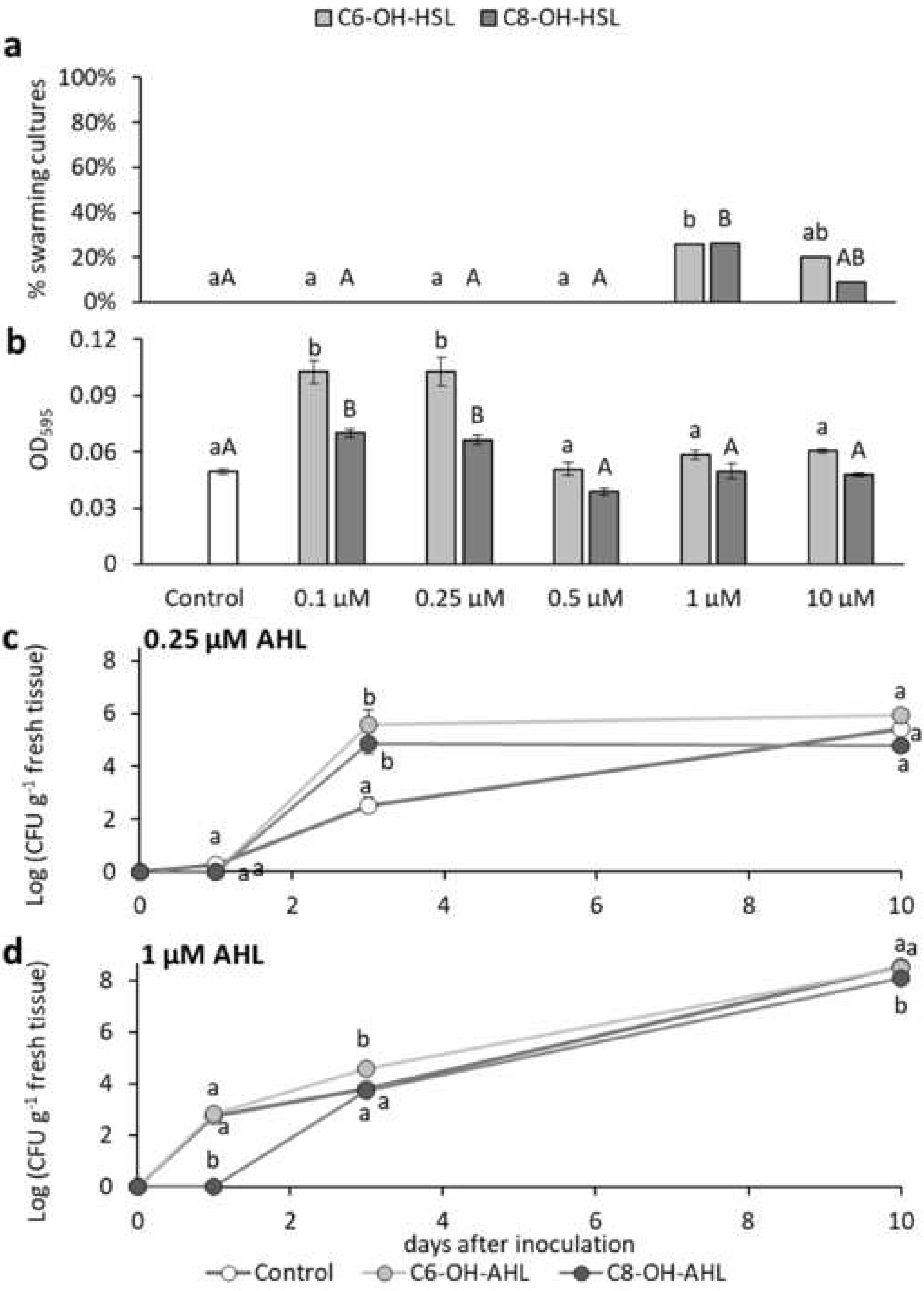
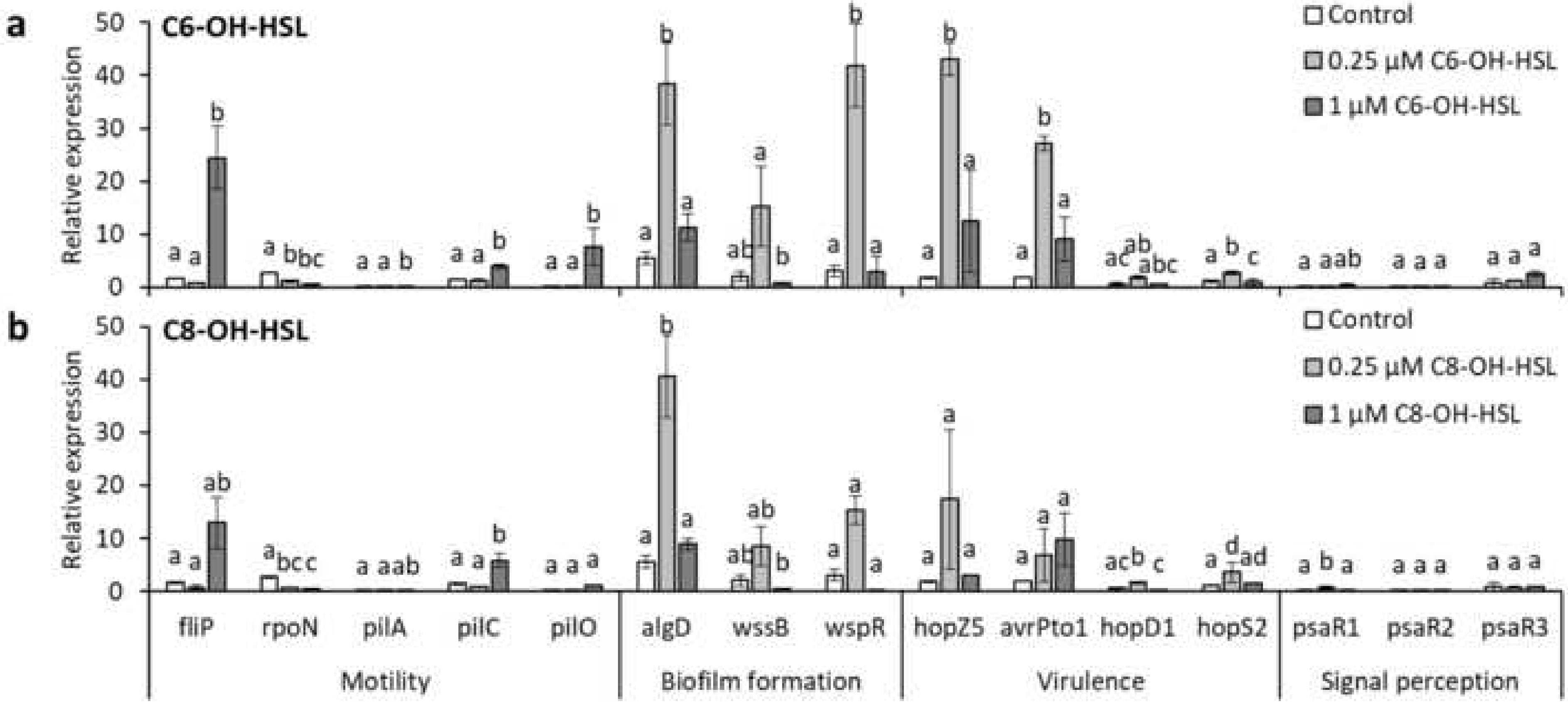

C6-OH-HSL
C8-OH-HSL
a
% swarming cultures
100%
80%
60%
40%
20%
0%
aA
a A
a A
a A
b B
ab
AB
b
OD$_{595}$
0.12
0.09
0.06
0.03
0
aA
b
B
b
B
a
A
a
A
a
A
Control
0.1 uM
0.25 uM
0.5 uM
1 uM
10 uM
c
0.25 μM AHL
Log (CFU g^{-1} fresh tissue)
8
6
4
2
0
b
b
a
a
a a
a
a
a
0
2
4
6
8
10
d
1 μM AHL
Log (CFU g^{-1} fresh tissue)
8
6
4
2
0
a
a
b
b
a
a
aa
b
0
2
4
6
8
10
days after inoculation
Control
C6-OH-AHL
C8-OH-AHL

Figure 5


a
C6-OH-HSL
Relative expression
0
10
20
30
40
50
☐ Control
▨ 0.25 µM C6-OH-HSL
■ 1 µM C6-OH-HSL
a a b
a b bc
a a b
a a b
a a b
a b a
ab a b
a b a
a b a
a b a
ac ab abc
a b c
a a ab
a a a
a a a
b
C8-OH-HSL
Relative expression
0
10
20
30
40
50
☐ Control
▨ 0.25 µM C8-OH-HSL
■ 1 µM C8-OH-HSL
a a ab
a bc c
a a ab
a a b
a a a
a b a
ab ab b
a a a
a a a
a a a
ac b c
a d ad
a b a
a a a
a a a
fliP
rpoN
pilA
pilC
pilO
algD
wssB
wspR
hopZ5
avrPto1
hopD1
hopS2
psaR1
psaR2
psaR3
Motility
Biofilm formation
Virulence
Signal perception

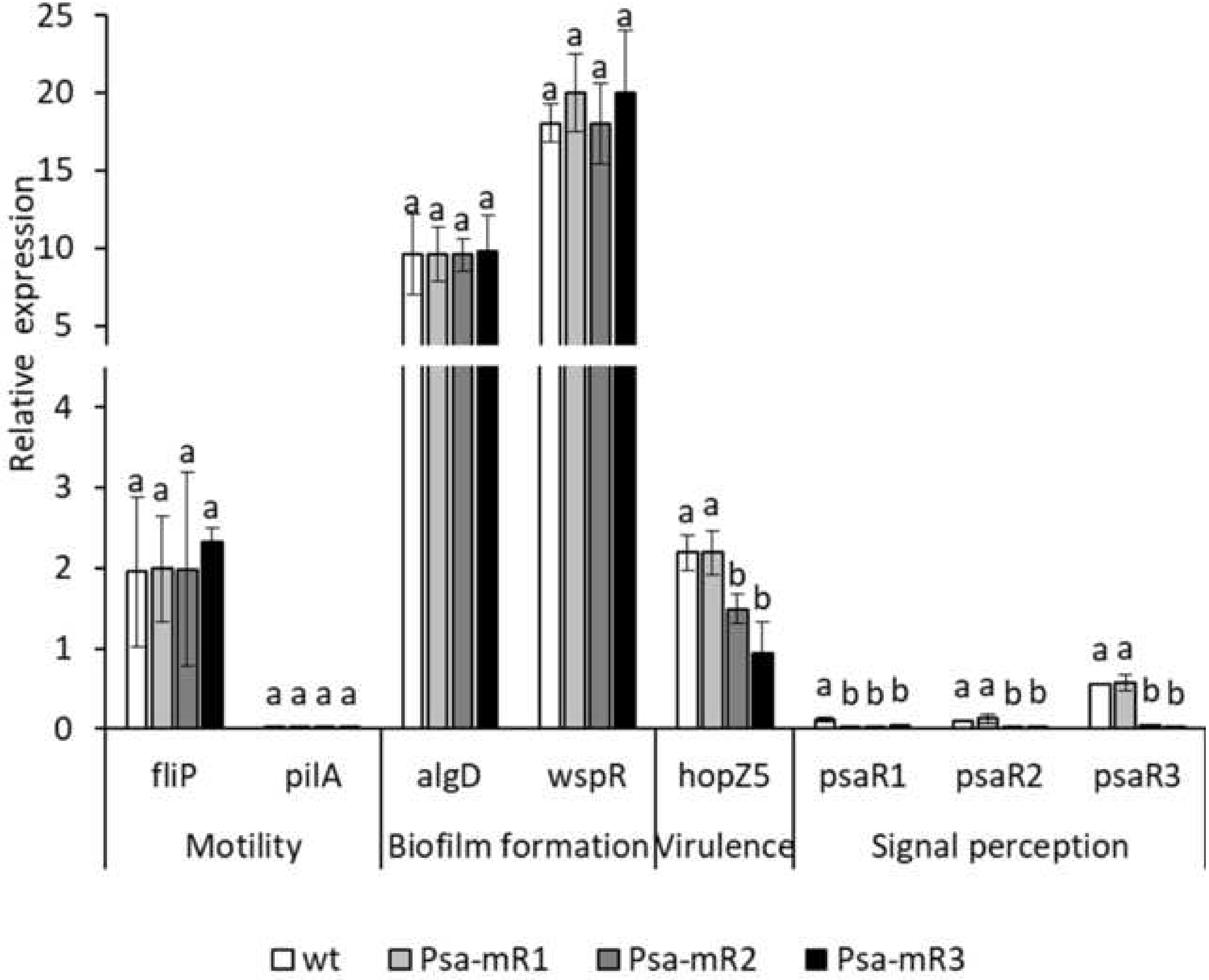

Relative expression
25
20
15
10
5
4
3
2
1
0
a a a a
a a a a
a a a a
a a a a
a a b b
a b b b
a a b b
a a b b
fliP
pilA
algD
wspR
hopZ5
psaR1
psaR2
psaR3
Motility
Biofilm formation
Virulence
Signal perception
□ wt
Psa-mR1
Psa-mR2
■ Psa-mR3

Figure 7

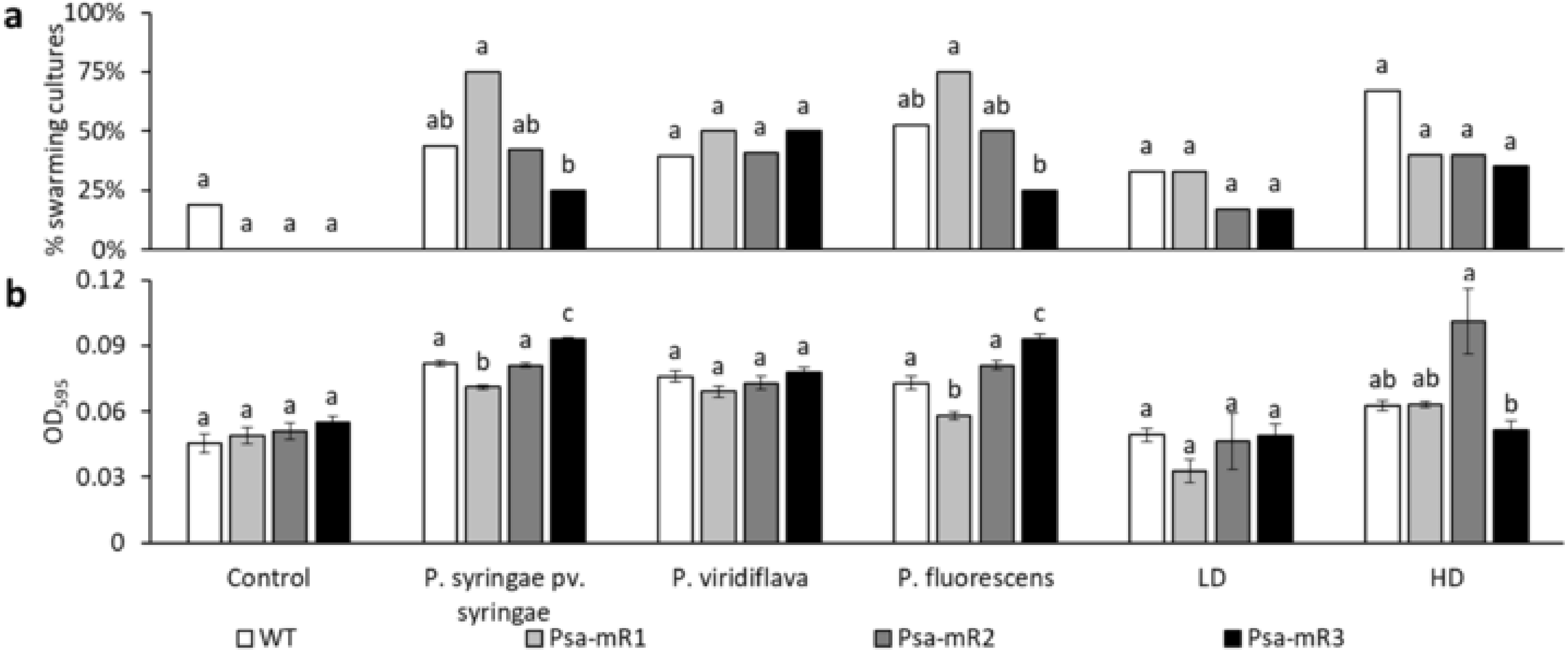

a
% swarming cultures
100%
75%
50%
25%
0%
a a a a
ab a ab b
a a a a
ab a ab b
a a a a
a a a a
b
OD595
0.12
0.09
0.06
0.03
0
a a a a
a b a c
a a a a
a b a c
a a a a
ab ab a b
Control
P. syringae pv. syringae
P. viridiflava
P. fluorescens
LD
HD
□ WT
▨ Psa-mR1
▨ Psa-mR2
■ Psa-mR3

Figure 8

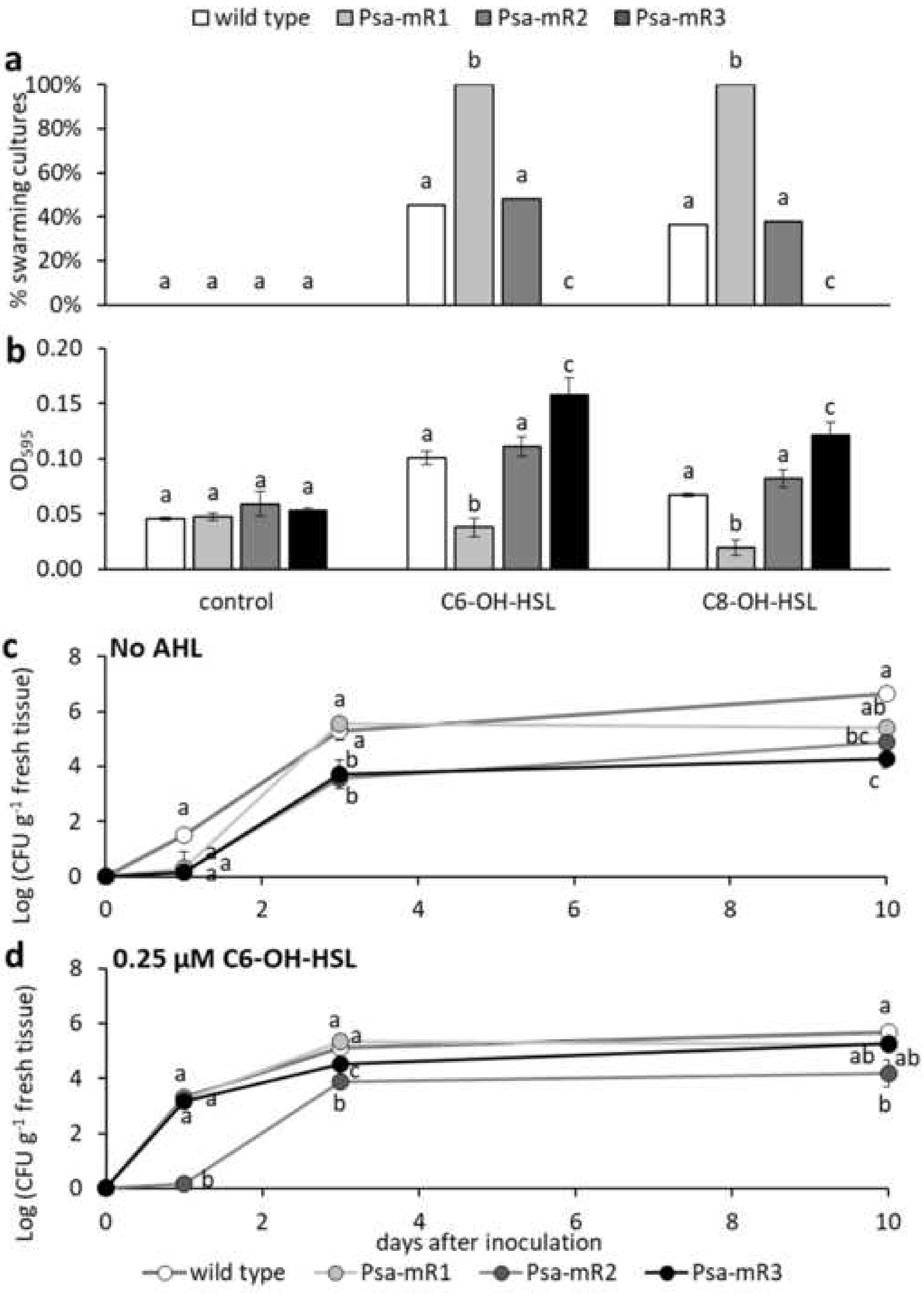
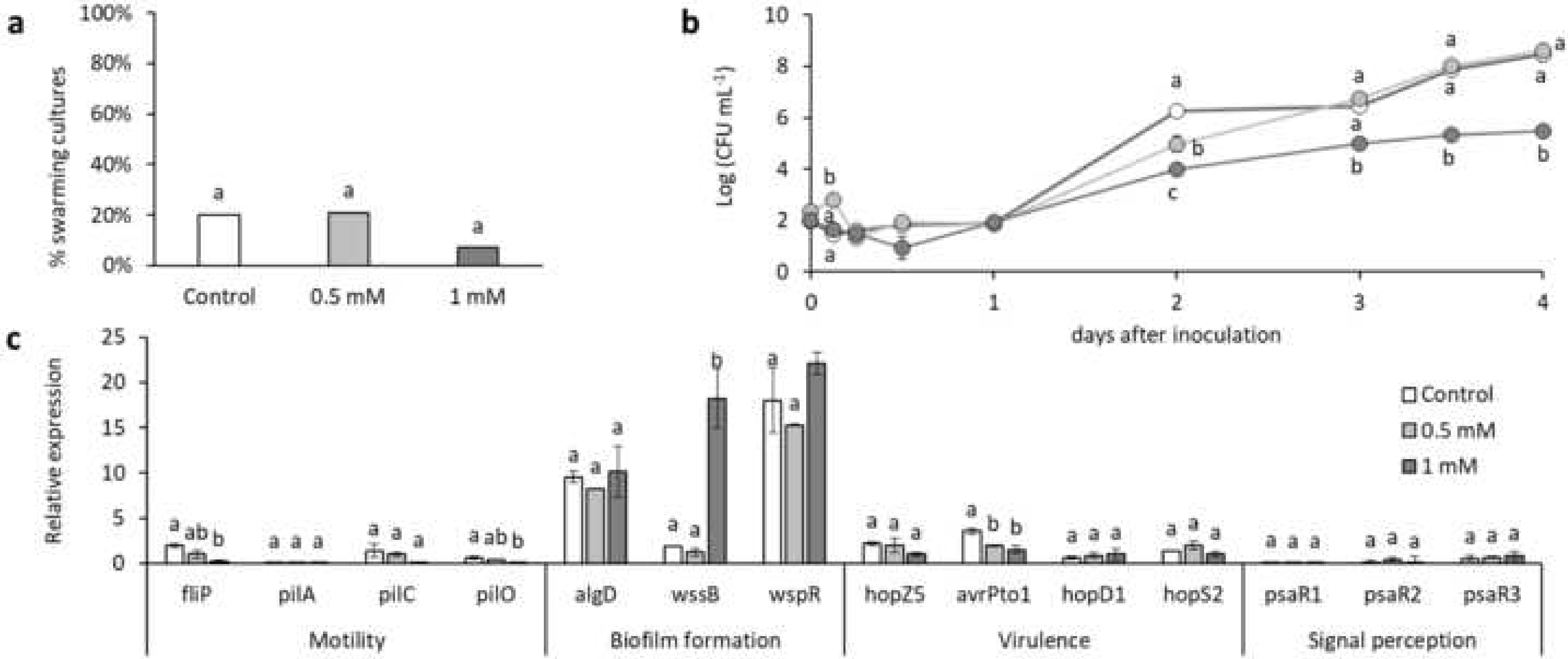

☐ wild type ▨ Psa-mR1 ▨ Psa-mR2 ■ Psa-mR3
a
% swarming cultures
100%
80%
60%
40%
20%
0%
a a a a
a b a c
a b a c
b
OD_{595}
0.20
0.15
0.10
0.05
0.00
a a a a
a b a c
a b a c
control
C6-OH-HSL
C8-OH-HSL
c
No AHL
Log (CFU g⁻¹ fresh tissue)
8
6
4
2
0
0
2
4
6
8
10
a
a
a
a
a
b
b
a
ab
bc
c
d
0.25 µM C6-OH-HSL
Log (CFU g⁻¹ fresh tissue)
a
a
a
b
a
a
c
b
a
ab
ab
b
days after inoculation
wild type
Psa-mR1
Psa-mR2
Psa-mR3

Figure 9


a
100%
80%
60%
40%
20%
0%
% swarming cultures
a
a
a
Control
0.5 mM
1 mM
b
10
8
6
4
2
0
Log (CFU mL^{-1})
0
1
2
3
4
days after inoculation
a
b
c
a
b
a
b
a
a
b
a
a
b
c
a
c
Relative expression
25
20
15
10
5
0
fliP
pilA
pilC
pilO
algD
wssB
wspR
hopZ5
avrPto1
hopD1
hopS2
psaR1
psaR2
psaR3
Motility
Biofilm formation
Virulence
Signal perception
☐ Control
▨ 0.5 mM
■ 1 mM

Supplementary Material 1

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Supplementary Material
OR_1_2.pdf

Supplementary Material 2