



Biosurfactants Produced by Phyllosphere-Colonizing Pseudomonads Impact Diesel Degradation but Not Colonization of Leaves of Gnotobiotic *Arabidopsis thaliana*

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

IMPORTANCE Many leaf-colonizing bacteria produce surfactants and are able to degrade aliphatic compounds; however, whether surfactant production provides a competitive advantage during leaf colonization is unclear. Furthermore, it is unclear if leaf colonizers take advantage of the aliphatic compounds that constitute the leaf cuticle and cuticular waxes. Here, we tested the effect of surfactant production on leaf colonization, and we demonstrate that the lack of surfactant production decreases the ability to degrade aliphatic compounds. This indicates that leaf surface-dwelling, surfactant-producing bacteria contribute to degradation of environmental hydrocarbons and may be able to utilize leaf surface waxes. This has implications for plant-microbe interactions and future studies.

KEYWORDS plant-microbe interactions, phyllosphere-inhabiting microbes, leaf wax, leaf cuticle, aliphatics, viscosin, massetolide A

Citation Oso S, Fuchs F, Übermuth C, Zander L, Daunaraviciute S, Remus DM, Stötzel I, Wüst M, Schreiber L, Remus-Emsermann MNP. 2021. Biosurfactants produced by phyllosphere-colonizing pseudomonads impact diesel degradation but not colonization of leaves of gnotobiotic *Arabidopsis thaliana*. *Appl Environ Microbiol* 87:e00091-21. <https://doi.org/10.1128/AEM.00091-21>.

Editor Maia Kivisaar, University of Tartu

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Received 15 January 2021

Accepted 12 February 2021

Accepted manuscript posted online

19 February 2021

Published 13 April 2021

The leaf cuticle is a hydrophobic barrier which consists of cutin, a polymer of very-long-chain aliphatics, interspersed and overlaid by very-long-chain monomeric aliphatics, cuticular waxes (1, 2). The cuticle reduces water loss, provides protection against UV radiation, and is the primary interface for plant microorganism and insect interactions (3–5). The cutin is a biopolymer which consists mainly of ω and midchain hydroxy and epoxy fatty acids (C_{16} to C_{18}) as well as glycerol (6–8). The cutin forms the structural backbone of the cuticle, as it is known to prevent mechanical damage. The cuticular waxes are the second major component of the leaf cuticle, mostly consisting of alkanes, alcohols, acids, and aldehydes with chain lengths between C_{16} and C_{32} . Cuticular waxes may also include secondary metabolites, such as flavonoids, triterpenoids, and phenylpropanoids (9). Cuticular waxes can be separated into two distinct waxes. The intracuticular wax within the cutin polymer is clearly distinct from the epicuticular wax, which is on the outer surface of the cutin polymer (10, 11). These differences thus affect the physical properties of the plant surfaces. The composition of the cuticular waxes is dependent on plant species and environmental conditions (12, 13). Wax monomers are very energy rich and a potential source of energy and carbon if they are bioavailable. However, it is still unclear whether bacteria are able to utilize these aliphatic compounds constituting the cuticle of living leaves as a source of carbon and whether surfactants facilitate their utilization.

Leaves are home to a wide variety of bacteria and can be covered by up to 5% bacterial biomass (14, 15). Many leaf surface-colonizing genera were previously shown to degrade hydrocarbons, e.g., *Rhodococcus* spp., *Sphingomonas* spp., *Pantoea* spp., *Methylobacterium* spp., and pseudomonads (16–19). Pseudomonads are common leaf colonizers and have many different ecological roles; e.g., many *Pseudomonas syringae* strains can be bona fide and host-specific pathogens (20), while others may act as antagonists against agents of plant disease (21, 22) or have unknown, tritagonistic (23) functions in the microbiota (24, 25). Pseudomonads have the ability to produce so-called biosurfactants in common (26). Biosurfactants are biologically produced amphiphilic molecules consisting of a hydrophilic head group and a hydrophobic moiety.

Leaf-colonizing pseudomonads produce cyclic peptide biosurfactants (26). Their ecophysiological role is not always clear, but it has been shown that pseudomonads may gain different fitness advantages by producing surfactants, including increasing survival during fluctuating humidity conditions on leaves (27) and increasing local water availability due to the hygroscopic nature of their surfactants (28). On agar plates, it has been shown that biosurfactants increase surface mobility by swarming, and it has been assumed that they may have similar functions on leaves (29).

In this study, we characterized the physiological effect of biosurfactants in four different pseudomonads that were isolated from leaves of spinach (*Pseudomonas* sp. strain FF1) or romaine lettuce (*Pseudomonas* sp. strains FF2, FF3, and FF4). Their biosurfactants were characterized using mass spectrometry, and their physical properties were analyzed. Furthermore, we investigated the ecophysiological functions of the biosurfactants for the bacteria. To that end, random insertion libraries were produced, and biosurfactant knockout mutants were identified. The knockout mutants were characterized in a series of experiments that investigated fitness changes *in vitro* and *in planta*.

RESULTS

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

Analysis of the 16S rRNA genes of all four isolates revealed that they are all members of the genus *Pseudomonas* and members of the *Pseudomonas fluorescens* lineage and subgroup (30). *Pseudomonas* sp. strain FF1 (PFF1) clusters closely with *Pseudomonas orientalis*, and *Pseudomonas* sp. strain FF2 (PFF2) clusters closely with *Pseudomonas extremaustralis*, while *Pseudomonas* sp. strain FF3 (PFF3) and *Pseudomonas* sp. strain FF4 (PFF4) cluster closely with *Pseudomonas paralactis* (Fig. 1). PFF1 and PFF2 are more closely related to each other than to PFF3 and PFF4. PFF3 and PFF4 are closely related.

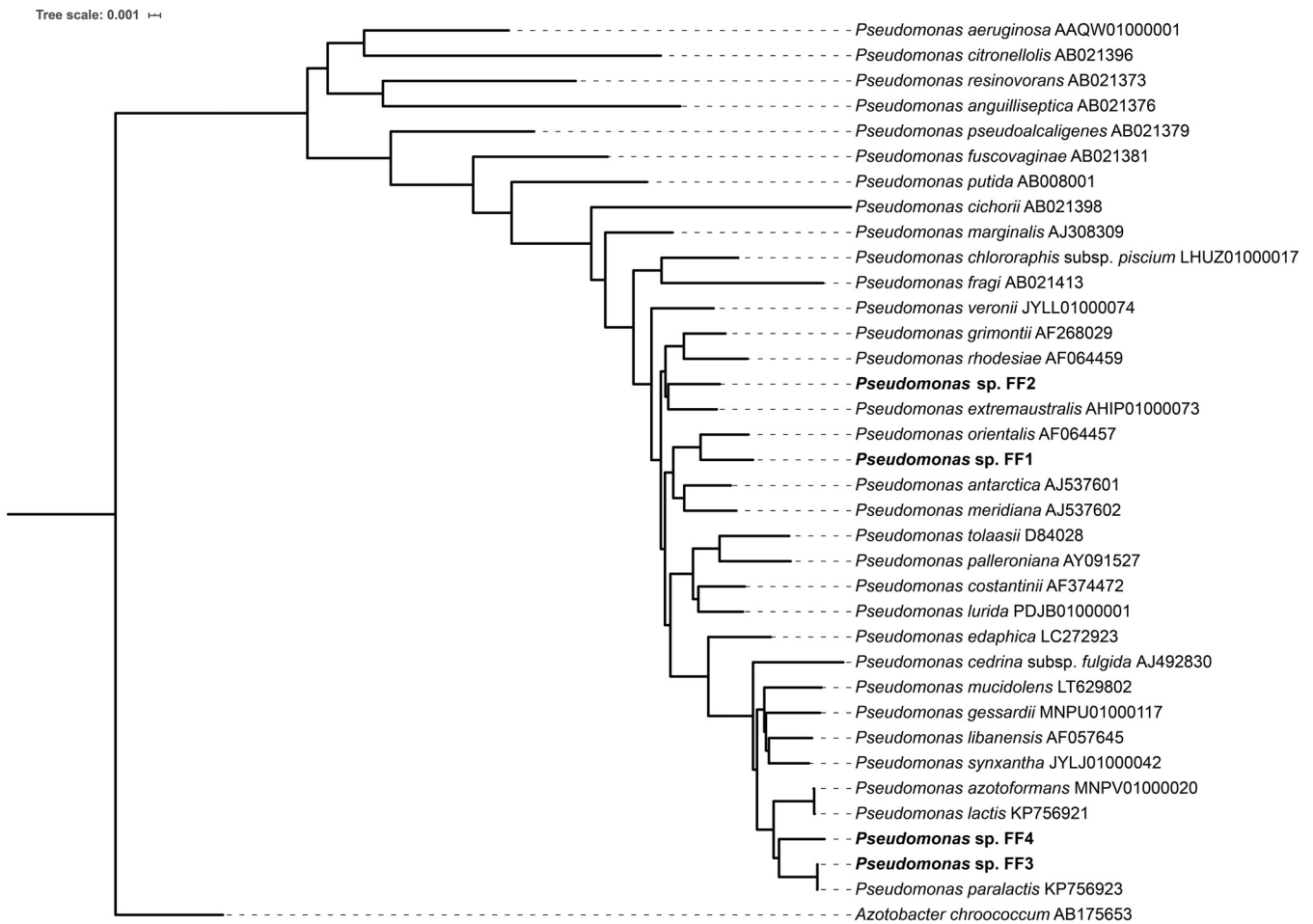


FIG 1 Phylogenetic placement of the four isolated pseudomonads. The newly sequenced isolates are highlighted in bold. NCBI accession numbers of the respective sequences are given after the species names. *Azotobacter chroococcum* was used as an outgroup.

Surfactant production of tested pseudomonads. All four wild-type pseudomonads were tested for their production of surfactants on agar plates using the atomized-oil assay (16, 31). All four strains produced clear halos where the reflection of the light by the oil, indicating production of surfactants (Fig. 2A to D). Similarly, the positive

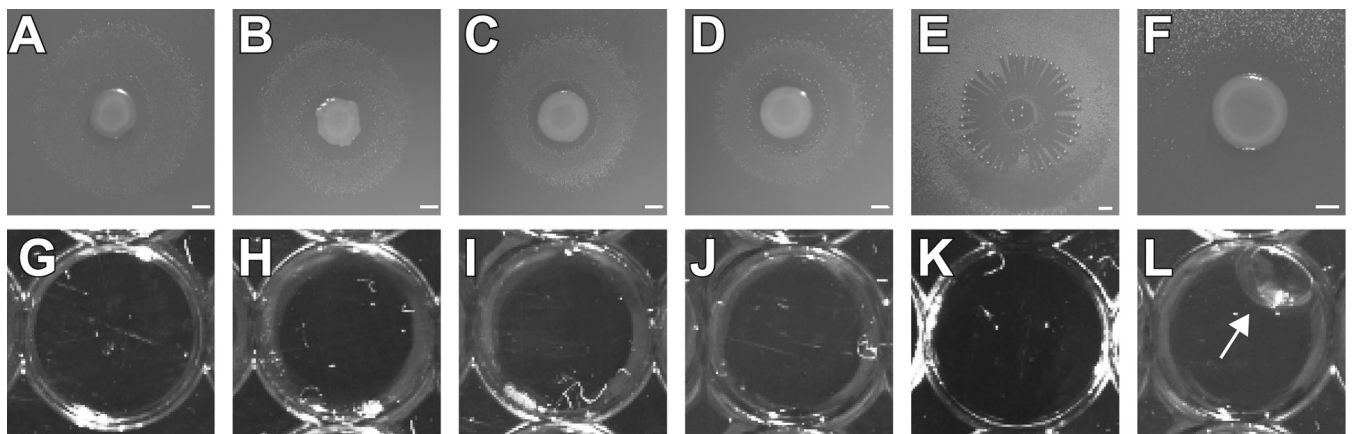


FIG 2 (A to F) Atomized-oil assays to demonstrate the production of surfactants. (A to D) Wild-type colonies of PFF1, PFF2, PFF3, and PFF4, respectively, exhibiting a halo indicative of surfactant production. (E) Tween 20. (F) *E. coli* DH5 α . (G to L) Drop collapse assays to demonstrate the production of surfactants. (G to J) Culture supernatants of wild-type PFF1, PFF2, PFF3, and PFF4, respectively, collapsed into oil, indicative of surfactant production. (K) Collapsed drop containing Tween 20. (L) Noncollapsed drop of *E. coli* culture supernatant (arrow).

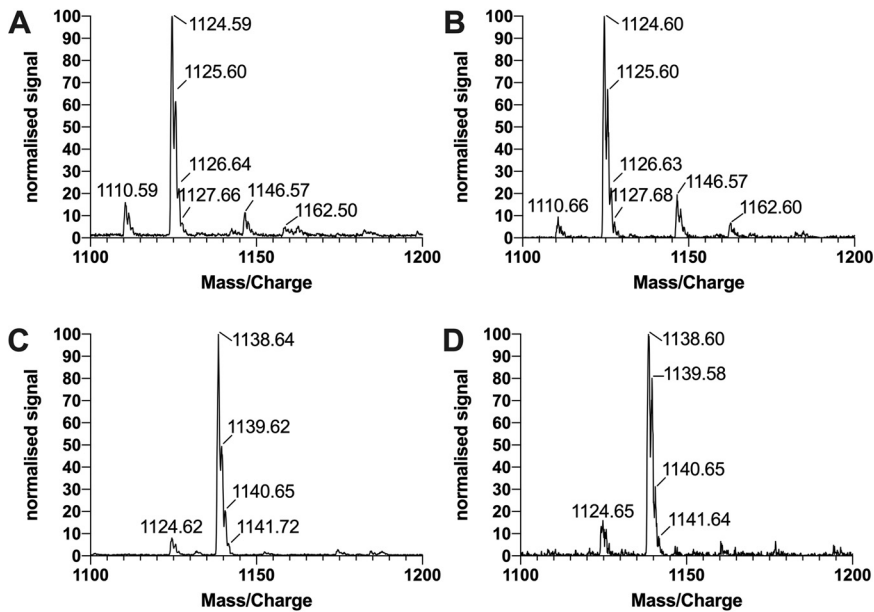


FIG 3 MS/MS spectra of extracted surfactants of PFF1, PFF2, PFF3, and PFF4 (A to D, respectively). PFF1 and PFF2 produce viscosin-like surfactants; PFF3 and PFF4 produce massetolide A-like surfactants. Spectra were normalized against the maximal intensity.

control, Tween 20, showed a halo (Fig. 2E), while the negative control, *Escherichia coli* DH5 α , lacked a halo (Fig. 2F). The drop collapse assay was used as a secondary test for surfactant production. All tested wild-type-culture supernatants collapsed into the engine oil (Fig. 2G to J). The collapse is due to a change in surface tension of the supernatant.

Mass-spectrometric analysis of surfactants. The analysis of surfactants harvested from the pseudomonads using liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization (ESI) in negative mode revealed that PFF1 and PFF2 produced the same compounds with a characteristic main peak at an m/z of 1,124.59 (Fig. 3A and B), which can be attributed to the deprotonated molecular ion $[M-H]^-$. The analogous pattern for the protonated molecular ion $[M+H]^+$ has been previously described for the cyclic lipopeptide viscosin with ESI in positive mode for detection (32, 33). Similarly, PFF3 and PFF4 share the same characteristic main peak at an m/z of 1,138.60 (Fig. 3C and D), and the analogous pattern has previously been described for the cyclic lipopeptide massetolide A (32).

Random Tn5 mutagenesis and mutant characterization. The surfactant-producing wild types were subjected to random insertion mutagenesis using the EZ-Tn5 transposon system. The screen resulted in a transposon mutant library with several hundred transposon mutants for each of the four isolates. We obtained 3 in 168, 4 in 1,100, 26 in 1,725, and 1 in \sim 200 surfactant negative mutants for PFF1, PFF2, PFF3, and PFF4, respectively. *Pseudomonas* genomes are between 6 Mbp and 6.5 Mbp; based on the previously published sizes of viscosin and massetolide A gene clusters (each \sim 30 kbp) (32, 34), we were expecting approximately 1 surfactant-negative mutant every \sim 200 to 220 clones, not considering essential genes. Each of the mutant libraries was screened with the atomized-oil assay for the lack of surfactant production mutants. For each strain, we selected one of the obtained surfactant mutants for further characterization studies (Fig. 4A to D). The drop collapse assay was conducted and confirmed the results of the atomized-oil assay (Fig. 4E to H). The insertion site of each mutant was determined by digesting the genomic DNA of the mutants and cloning it into pUC19 before selecting for kanamycin resistance encoded in the transposon (see Table S1 in the supplemental material).

The PFF1 mutant we investigated carried an insertion in a gene with 97% similarity

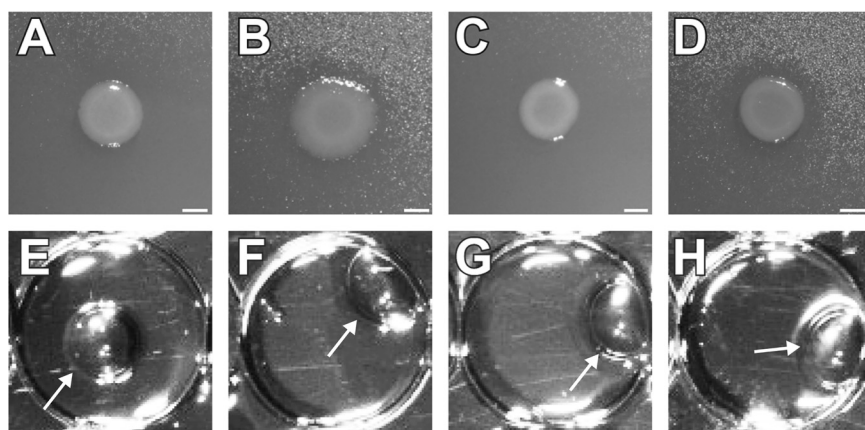


FIG 4 (A to D) Atomized-oil assay to demonstrate the production of surfactants. Tn5 insertion mutant colonies of PFF1::ezTn5-visB, PFF2::ezTn5-visB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB, respectively, lacking a halo indicative of surfactant production. (E to H) Drop collapse assays to demonstrate the production of surfactants. Culture supernatants of Tn5 insertion mutants PFF1::ezTn5-visB, PFF2::ezTn5-visB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB, respectively, showing a beaded bubble swimming on top of the oil, indicative of the lack of surfactants. The noncollapsed droplets are indicated by arrows.

to a nonribosomal peptide synthetase in *P. orientalis* F9 (GenPept accession number [AUZ46831](#) [locus tag BOP93_14875] (21), which has 80% peptide similarity to the *viscB* gene of *P. fluorescens* SBW25 (UniProtKB ID [C3K9G2](#)) (34, 35). The PFF2 mutant carried an insertion in a gene with 86% similarity to the *viscB* gene (GenPept no. [CAY48788.1](#)) of *P. fluorescens* SBW25. Therefore, these mutants are designated PFF1::ezTn5-*viscB* and PFF2::ezTn5-*viscB*, respectively. The *viscB* gene encodes a nonribosomal peptide synthetase that, in conjunction with *viscA* and *viscC*, produces the cyclic lipopeptide biosurfactant viscosin (34). The PFF3 Tn5 transposon mutant carried an insertion in a gene with 99% similarity to the *massB* gene in *Pseudomonas fluorescens* SS101 (GenPept no. [ABH06368.2](#)). The PFF4 Tn5 transposon mutant carried an insertion in a gene with 95% similarity to the *massB* gene in *Pseudomonas fluorescens* SS101 (32). The *massB* gene is part of the massetolide A synthesis gene cluster. Therefore, the mutants were designated PFF3::ezTn5-*massB* and PFF4::ezTn5-*massB*.

After the surfactant extraction from mutant-inoculated agar plates, no surfactants could be detected (Fig. 5A to D). The effect of the transposon insertions and the lack of surfactant production was tested in shaking liquid cultures in two different conditions, either KB complex medium (see Fig. S1A) or M9 minimal medium supplemented with glucose as the sole source of carbon (see Fig. S1B). None of the tested insertion mutants exhibited significantly changed doubling times under the two sets of conditions tested.

Growth on diesel oil or dodecane as the sole carbon source. To investigate if the lack of surfactant production could impact the ability of the pseudomonad strains to degrade alkanes, the different wild types and transposon mutants were grown on Bushnell-Haas broth with diesel as the sole carbon source. This experiment revealed that all surfactant mutants, even though they were still able to grow on diesel, had a reduced growth rate and a reduced final optical density after up to 21 days of growth (Fig. 6). No growth could be observed on Bushnell-Haas broth without a carbon source for either the wild types or the surfactant mutants. In general, the growth on diesel oil was slower than growth on complex medium or minimal medium supplemented with glucose as the sole carbon source and was better described by a linear function than an exponential growth function. By supplementing knockout mutants with biosurfactants harvested from respective wild-type strains or with the synthetic surfactant Tween 20, growth on diesel could be complemented, in part or completely, compared

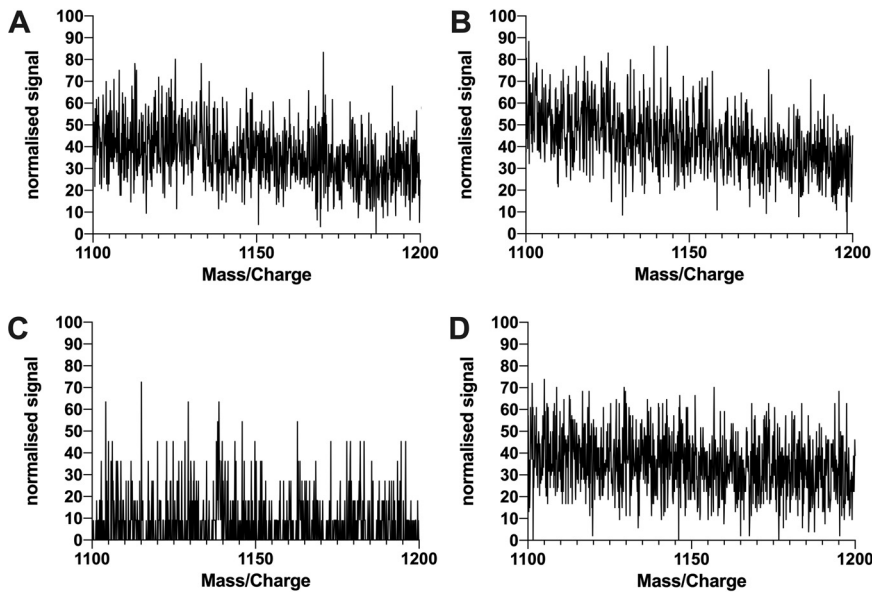


FIG 5 Knockout mutants show no sign of surfactant production. MS/MS spectra of extracts of PFF1::ezTn5-viscB, PFF2::ezTn5-viscB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB (A to D, respectively), are shown. None of the random knockout mutants produced detectable surfactant peaks at the corresponding wild-type *m/z* values. Spectra were normalized against the maximal intensity.

to the wild type. The knockout mutants were not able to grow on surfactants alone to a degree that explains the increased growth on diesel (Fig. S2).

While growing on dodecane, surfactant production had a less dramatic effect than that during growth on diesel (Fig. 7 and Table S3). The overall growth was lower and the maximal optical density at 600 nm (OD₆₀₀) of all tested strains was more than an

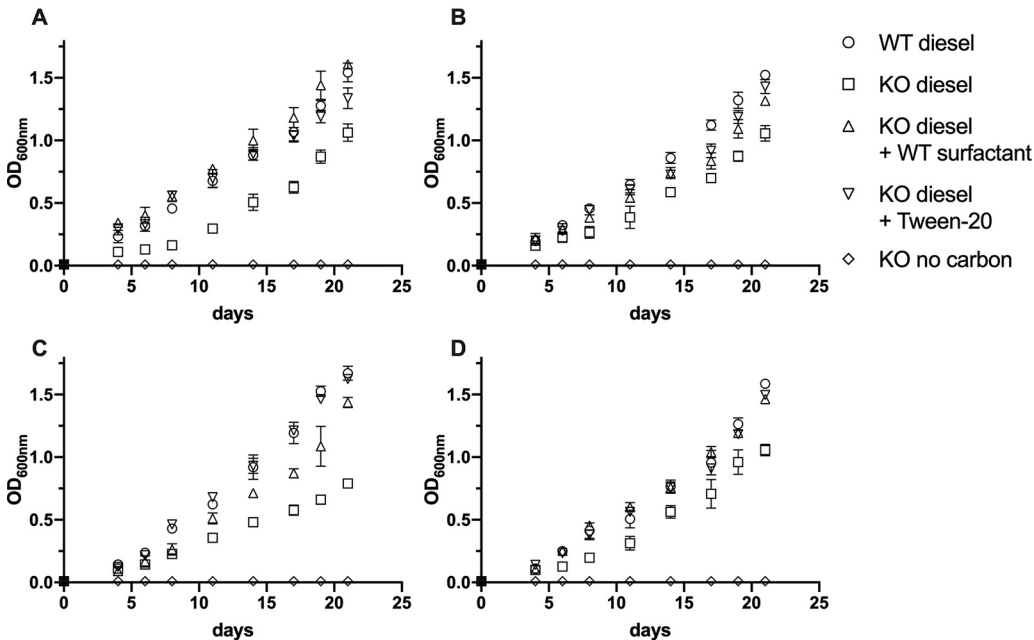


FIG 6 Utilization of diesel by biosurfactant knockout mutants and wild types. (A) PFF1; (B) PFF2; (C) PFF3; (D) PFF4. Each wild type and knockout mutant was grown in Bushnell-Haas broth supplemented with diesel as the sole source of carbon (circles and squares, respectively). Knockout mutants were complemented with either wild-type surfactant (triangles) or Tween 20 (inverted triangles) or were incubated with no additional carbon source (diamonds). The statistical analysis can be found in Table S3. Error bars depict the standard deviations of the means. Experiments were performed in triplicate.

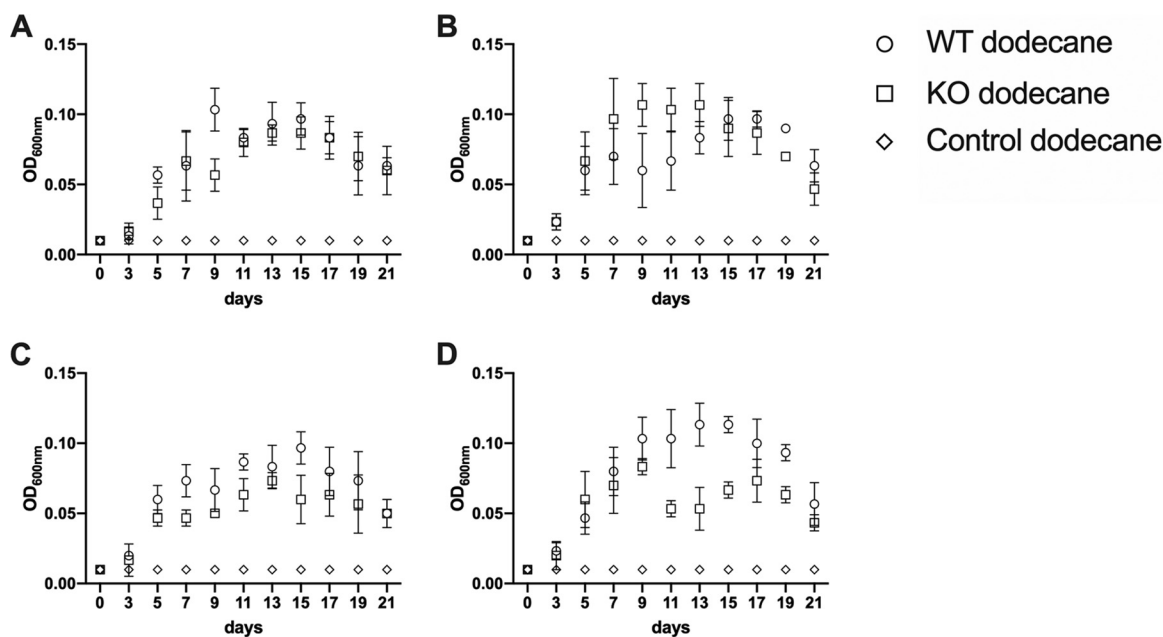


FIG 7 Growth of *Pseudomonas* knockout strains on dodecane as the sole carbon source. Bushnell-Haas broth (BHB) supplemented with dodecane was inoculated with either wild-type strains (circles) or surfactant knockout mutant (squares) or was left noninoculated (diamonds). (A) Growth of PFF1 and the respective surfactant mutant. The growth of the surfactant mutant was significantly lower than that of the wild type on days 5 and 9. (B) Growth of PFF2 and the respective surfactant mutant. From day 7 to day 13, the growth of the surfactant mutant was significantly lower than that of the wild type. (C) Growth of PFF3 and the respective surfactant mutant. The growth of the surfactant mutant was significantly lower than that of the wild type on days 7, 11, and 15. (D) Growth of PFF4 and the respective surfactant mutant. From day 9 to day 19, the growth of the surfactant mutant was significantly lower than that of the wild type. The statistical analysis can be found in Table S3. Error bars depict the standard deviations of the means from three replicates.

order of magnitude lower than during growth on diesel, and the maximal OD₆₀₀ was reached after 9 to 13 days. After 21 days, the optical densities were already markedly reduced, and the cultures were in their death phase. For all wild-type strains and combinations with all mutants but PFF2, we were able to detect periods where the wild type achieved significantly higher optical densities than the knockout mutants. The PFF2 surfactant knockout mutant reached its maximal optical density earlier than the PFF2 wild type.

Fitness *in planta*. To investigate changes in the ability of the transposon mutants to colonize leaf surfaces, the mutants were coinoculated with the respective wild types by airbrushing. Whole above-ground plant material was sampled daily for 6 days, and numbers of CFU of the wild type and transposon mutants were determined (Fig. 8). The initial bacterial densities were similar between wild types and knockout mutants. Wild types (PFF1, PFF2, PFF3, and PFF4) and corresponding mutants (PFF1::ezTn5-viscB, PFF2::ezTn5-viscB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB) colonized *Arabidopsis* at similar rates. PFF1, PFF2, and their mutants reached approximately 10⁷ CFU per g of plant weight, whereas PFF3, PFF4, and their mutants reached approximately 10⁶ CFU per g of plant weight. Thus, no differences between the plant colonization of wild types and mutants were found. Furthermore, growth *in planta* of all strains was tested individually, and no significant differences in plant colonization could be determined (Fig. S3).

DISCUSSION

All four pseudomonads isolated from either spinach or romaine lettuce leaf material (36) belong to the fluorescent pseudomonads (37). PFF1 and PFF2 are phylogenetically more closely related to each other than to PFF3 and PFF4. PFF3 and PFF4 are very closely related. All four strains produced surfactants on agar plates and in liquid

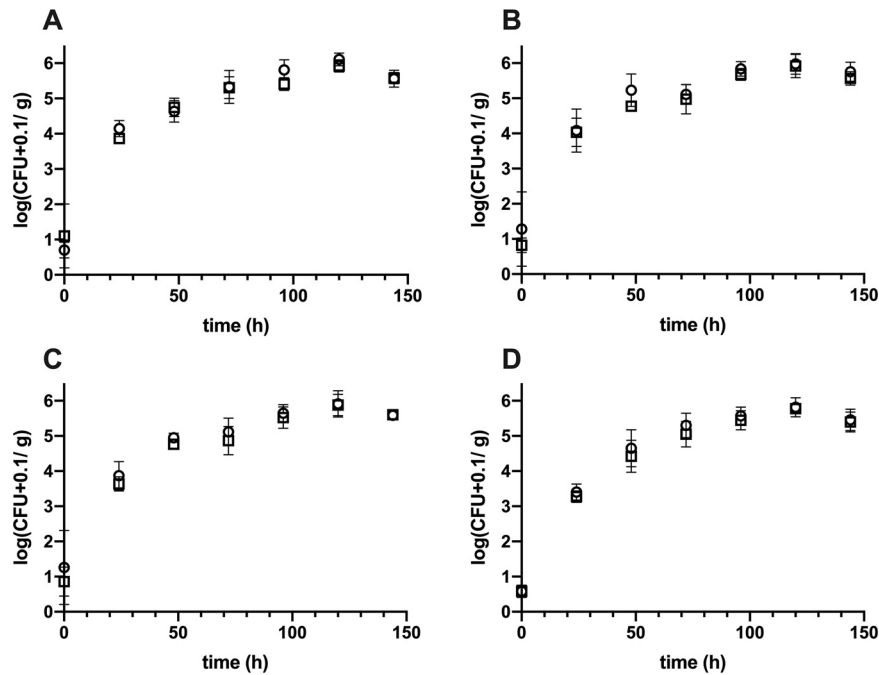


FIG 8 *In planta* competition of wild types (circles) and mutants (squares). (A) PFF1 versus PFF1::ezTn5-visB; (B) PFF2 versus PFF2::ezTn5-visB; (C) PFF3 versus PFF3::ezTn5-massB; (D) PFF4 versus PFF4::ezTn5-massB. Symbols represent the mean number of CFU on five plants per measurement. The statistical analysis can be found in Table S3. Error bars depict the standard deviations of the means. Experiments were performed in quintuplicate.

culture, as shown by the atomized-oil assay and the drop collapse assay. As the ability to produce surfactants is widely distributed in the genus *Pseudomonas*, this result was not surprising (38, 39). The relatedness of the strains is also reflected in the surfactants that each of the strains produces: PFF1 and PFF2 produce the viscosin-like surfactants, while PFF3 and PFF4 produced massetolide A-like surfactants. The production of viscosin and massetolide A by pseudomonads has been demonstrated previously (32). Both viscosin and massetolide A are the product of nonribosomal peptide synthetase genes. Viscosin production depends on a gene cluster which encompasses the genes *viscA*, *viscB*, and *viscC* and which spans approximately 32 kb (34). Massetolide A production depends on a gene cluster which encompasses the genes *massA*, *massB*, and *massC* and spans approximately 30 kb (32).

To further investigate the ecological function of the surfactants in the leaf-colonizing pseudomonads, random Tn5 transposon insertion mutants were produced and further characterized. The screen yielded complete loss of surfactant production mutants for every strain, indicating that each strain encodes only one surfactant that is active during the selection conditions. The insertion sites were mapped to genes which matched previously characterized nonribosomal peptide synthetase clusters responsible for surfactant production and which matched the surfactants that were identified using mass spectrometry. PFF1 and PFF2 knockout mutants were mapped to *viscB* gene homologues, and PFF3 and PFF4 knockout mutants were mapped to *massB* gene homologues (32, 34).

The assumption that only one surfactant is produced by each strain was corroborated by a sequence of experiments during which the surfactant mutants consistently failed to produce signs of surfactant production independent of their growth conditions. The surfactant mutants failed to produce halos in the atomized-oil assay, and the culture supernatant did not collapse into motor oil in the drop collapse assay. Mass-spectrometric analysis of the knockout mutants showed that the production of

surfactants was completely abolished, and no detectable peak pattern was found after the surfactant extraction procedure (Fig. 5).

Despite the loss of surfactant production and the additional burden of expressing the kanamycin resistance gene from the Tn5 transposon, the insertions had no detectable fitness effects in either complex KB medium or minimal M9 medium supplemented with glucose. In shaking liquid cultures, surfactants did not provide critical functions for growth (Fig. S1). We hypothesize that surfactants may enable bacteria to utilize parts of the plant cuticle as a source for carbon. To that end, we tested the strains' abilities to utilize diesel as a proxy for aliphatic compounds also found in cuticular waxes. A clear difference in the abilities of wild types and mutants to utilize diesel for growth was demonstrated (Fig. 6). Even though growth was not completely abolished, it was significantly reduced (Table S2). Due to the size of the nonribosomal peptide synthetase genes, it was not possible to construct rescue mutants. However, we attempted to complement the reduced ability of the knockout mutants to degrade diesel oil by adding harvested wild-type surfactant or Tween 20 to growing cultures. Indeed, both surfactants were able to complement the growth phenotype either in part or completely (Fig. 6 and Table S2), indicating that the lack of surfactants was the causal reason for reduced growth. Despite the chain length differences between the diesel (40) and the alkane monomers in waxes of leaf cuticles (2), both aliphatic mixtures contain similar monomers. It is thus not unthinkable that, under nutrient-limiting conditions, the *Pseudomonas* strains tested here are able to utilize aliphatic components of leaf cuticles in a surfactant-dependent manner. However, we failed to provide a final proof of this relationship.

To investigate the role of the surfactants during plant colonization, we inoculated axenically grown *Arabidopsis* with mixtures of wild types and knockout mutants or with individual strains. During coinoculation with their respective wild types onto axenic *Arabidopsis*, no fitness disadvantages for the knockout mutants were detected. This might be a consequence of the surfactant acting as a public good that increases the fitness of wild types and coinoculated mutants alike (41). However, single-strain inoculations also did not result in a diminished ability of the knockout mutants to colonize *Arabidopsis*. This is in contrast to previous experiments that demonstrated that surfactants do indeed have a positive effect on plant colonization (27). It is noteworthy that the experimental setup used in our study was markedly different, including a different plant host as well as incubation conditions under constant relative humidities, whereas previously, it was shown that fluctuating humidities are a prerequisite to result in a fitness advantage. Therefore, it might still be possible that the surfactants in the strains tested here impact plant colonization, for example, under fluctuating relative humidities, by increasing mobility of the strains on the phylloplane (42, 43) or increasing permeability of leaf cuticles (44). Furthermore, the antimicrobial activity of many surfactants may provide fitness advantages in microbial communities (43).

Conclusion. The experiments reported here demonstrated that the biosurfactants produced by four different leaf-colonizing pseudomonads impacted their ability to degrade aliphatic compounds. However, the ability to produce biosurfactants had no measurable impact on the ability of the strains to colonize axenic *Arabidopsis* leaves in competition or after individual strain inoculations. We gathered additional evidence that the bacteria may utilize aliphatic compounds originating from leaf cuticles but failed to conclusively demonstrate a relationship between surfactant production and leaf colonization ability. Future studies will have to be performed to address this hypothesis.

MATERIALS AND METHODS

Bacterial strains used in this study. Bacteria used in this study were *Pseudomonas* sp. strain FF1 (PFF1), PFF2, PFF3, and PFF4 (45); all pseudomonads were kind gifts from Adrien Burch and Steven Lindow (University of California, Berkeley). *E. coli* Stellar (Lucigen) was used for cloning. PFF1 was isolated from spinach, and PFF2, PFF3, and PFF4 were isolated from romaine lettuce. Pseudomonads were routinely grown on liquid King's B (KB) (20 g proteose peptone, 1.15 g K₂HPO₄, 1.5 g MgSO₄·7H₂O, 10 g glycerol per liter [pH 7]; for agar medium [KBA], 15 g agar was added per liter) or lysogeny broth (LB) (5 g

yeast extract, 10 g tryptone, 10 g NaCl per liter [pH 7]; for agar medium, 15 g agar was added per liter). *E. coli* was routinely grown on LB and LBA. For *in planta* competition experiments, spontaneous streptomycin-resistant mutants of the wild-type *Pseudomonas* were selected (46). Where appropriate, the media were supplemented with kanamycin ($50 \mu\text{g ml}^{-1}$) or streptomycin ($50 \mu\text{g ml}^{-1}$).

16S rRNA gene sequencing. To determine the phylogeny of the strains, their 16S rRNA genes were amplified from genomic DNA that was extracted using the NucleoSpin microbial DNA kit (Macherey-Nagel) following the manufacturer's recommendations. A PCR using KAPA2G Fast 2 \times ready mix with dye (Kapa) was performed using the manufacturer's recommendation, with 1 μl of the genomic and 16S rRNA gene-targeting primers SLK8-F 5'-AGAGTTTGATCATGGCTCAGAT-3' and SRK1506-R 5'-TACCTTGTTACGACTTCAACCC-3'. The resulting ~ 1.5 -kbp fragments were sequenced (Eurofins Genomic) and then curated and assembled using Geneious prime (Geneious). The assembled fragments were uploaded to ezbiocloud (47), and the 30 best matches of organisms that were validly named were recovered for each of the four strains. Additional *Pseudomonas* 16S sequences and outgroup sequences were recovered from the silva database (48). All sequences were compiled into a FASTA file and aligned and visualized using the FastME/OneClick option of ngphylogeny.fr (49). The resulting tree was imported into iTOL, edited for publication, and then exported (50).

Preparation of electrocompetent pseudomonads. Electrocompetent pseudomonads were produced as explained elsewhere (51). Briefly, bacteria were grown overnight in 6 ml KB in a shaking incubator at 25°C. Three milliliters of the overnight culture was then used to inoculate 100 ml KB, which was incubated at 25°C in a shaking incubator until the culture reached a mid-exponential-growth-phase OD_{600} of approximately 0.6. The culture was then split into 50-ml aliquots and cooled on ice for 30 min. Bacteria were then harvested by centrifugation at $6,000 \times g$ and 4°C for 10 min. The supernatant was discarded, and the aliquots were washed twice with 50 ml ice-cold sterile water. Then the aliquots were washed in 25 ml ice-cold water and combined again. After a final centrifugation, the cell pellet was resuspended in 250 μl sterile 10% glycerol and distributed in 50- μl aliquots that were stored at -80°C .

Random transposon mutagenesis. Random knockout mutants were produced using the EZ::Tn5TM <KAN-2> Tnp transposome kit (Epicentre) following the manufacturer's recommendations. In brief, 50 μl electrocompetent pseudomonads were thawed on ice and 1 μl Tn5 transposome and 1 μl endonuclease inhibitor were mixed with the cells. The mixture was incubated for 5 min on ice before the cells were pipetted into a prechilled 0.1-cm-gap electroporation cuvette. A Gene Pulser (Bio-Rad) was used to pulse the cells (2.5 kV, 200 Ω , 25 μF). Immediately after that, 1 ml SOC (SOB is 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 250 mM KCl per liter [pH 7]; SOC is SOB supplemented with 5 ml 2 M MgCl_2 and 20 ml 1 M glucose) was added, and the cells were incubated for 1 h at 30°C and 150 rpm. Transposon insertion mutants were selected on minimal medium agar plates (15 ml glycerol, 5 g L-glutamine, 1.5 g K_2HPO_4 , 1.15 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g agar per liter [pH 7]) supplemented with kanamycin. Minimal medium was used to prevent the growth of auxotrophic mutants. Transposon mutants could be detected after 2 days.

To determine the site of transposon integration, genomic DNA of knockout mutants was isolated using the Isolate II kit (Bioline). Genomic DNA was cut using KpnI (New England Biolabs) or EcoRI and ligated into similarly digested and dephosphorylated vector pUC19 (New England Biolabs) using T4 ligase (New England Biolabs) following the recommendations of the manufacturer. A 5- μl portion of each ligation mixture was transformed into chemically competent *E. coli* Stellar using the manufacturer's recommendations. Clones harboring plasmids containing the transposon were selected on LB supplemented with kanamycin. Inserts of the plasmids were sequenced using the transposon-specific primer kan2_RP-1 (5'-GCAATGTAACATCAGAGATTTGAG-3'). Sequencing results were compared to the NCBI database using NCBI BLAST restricted to the genus *Pseudomonas* (52).

Screens for surfactant production. To screen for surfactant production, the atomized-oil assay was performed (31). To that end, agar plates containing transposon mutants were sprayed with hydrophobic dodecane using an airbrush. Bacterial colonies that produced surfactants resulted in a halo around the colony where the surfactant in the agar changed the surface angle of oil droplets on the surface. Colonies that lacked this characteristic halo were further characterized. Presumptive surfactant mutants were tested in the drop collapse assay as described previously (16). Briefly, 2 μl of Magnatec 10W-40 oil (Castrol) was pipetted into each well of a 96-well plate lid (Corning Incorporated) and allowed to equilibrate for 2 h to ensure that each well was evenly coated. Bacterial overnight cultures were centrifuged at $2,600 \times g$ for 10 min. Five microliters of the culture supernatant was pipetted into the center of an oil-filled well. Drops that collapsed into the oil, i.e., decreased their contact angle, were positive for surfactant production, while drops that remained intact and stayed on top of the oil were negative for surfactant production. All experiments were performed in at least 8 biological replicates.

Extraction of surfactants. Bacterial strains were grown as crude streaks on five separate KBA plates for 48 h at 25°C. Afterwards, bacterial biomass was harvested using 5 ml of sterile water per plate, and the cell suspensions of all 5 plates were combined in a 50-ml centrifugation tube. Ethyl acetate (25 ml) was added to the suspension, and the tube was vortexed for 3 min. The mixture was then centrifuged for 10 min at $1,000 \times g$ to facilitate separation of the aqueous and organic phase. The organic phase was recovered using a glass pipette and transferred to a glass vessel before the ethyl acetate was evaporated off under constant nitrogen flow. The result was resolved in ethanol and sterile filtered through a 0.22- μm filter. The filtered solution was then dried under constant nitrogen flow and weighed before it was resuspended to $5 \mu\text{g ml}^{-1}$ in ethyl acetate.

Mass-spectrometric analysis. Mass-spectrometric analysis of the biosurfactants was performed using a QTRAP 4500 (Applied Biosystems, AB Sciex) triple-quadrupole mass spectrometer, operated in

negative electrospray ionization (ESI)-Q1 scan mode. The surfactant solution with a concentration of $5 \mu\text{g ml}^{-1}$ was injected via a syringe pump set to a flow rate of $10 \mu\text{l min}^{-1}$ directly into the MS. The analytes were detected in negative mode within a mass-over-charge (m/z) range of 1,000 to 1,200.

Plant growth and *in planta* experiments. *Arabidopsis thaliana* was grown axenically as described previously (53). Briefly, *Arabidopsis* seeds were sterilized in a 1.5-ml Eppendorf tube by adding 1 ml 70% ethanol and 0.1% Triton X-100. The seeds were vortexed and then incubated for 1 min. The supernatant was removed by pipetting, followed by the addition of 1 ml of 10% bleach and $10 \mu\text{l}$ of 0.1% Triton X-100 for 12 min. After the bleach was removed, the seeds were rinsed three times with 1 ml of sterile distilled water and stratified for 48 h at 4°C. Stratified seeds were pipetted onto Murashige and Skoog agar (MS agar; 2.2 g of Murashige and Skoog medium including vitamins [Duchefa] and 10 g plant agar [Duchefa] per liter of Milli-Q water; pH 5.8) with filled 200- μl pipette tips that were shortened by 1 cm to allow the plant's roots to easily pass the tip. The tips were placed pointed end first into an MS agar plate. The seeds were germinated for 7 days under short-day conditions (11 h day/13 h night). After the germination period, the seedling-filled tips were transferred to autoclaved Magenta GA-7 (bioWORLD) plant culture boxes filled with 90 g finely ground zeolite clay (Purfit Clay Litter; Vitapet) and 60 ml MS medium. Four seedlings were transferred into each Magenta box, and the plants were grown for an additional 3 weeks under short-day conditions (11 h day/13 h night; chamber set to 85% relative humidity). To prepare bacterial inocula, bacteria were cultured on LB broth overnight. Bacteria were then harvested by 10 min centrifugation at $2,600 \times g$ and washed with $1 \times$ phosphate-buffered saline (PBS; 0.2 g liter^{-1} NaCl, $1.44 \text{ g liter}^{-1}$ Na_2HPO_4 , and $0.24 \text{ g liter}^{-1}$ KH_2PO_4). Bacteria were resuspended to an OD_{600} of 0.5 and then serially diluted to and OD_{600} of 0.00005. For competition experiments, wild-type and surfactant knockout strains were mixed at a ratio of 1:1. A 100- μl portion of the mixtures or the monocultures were inoculated onto 3-week-old *Arabidopsis* plants using a T-180 airbrush (KKmoon).

Bacteria were recovered by harvesting the leaf material of individual plants and placing the samples in a 1.5 ml Eppendorf vial. The plants were weighed, and 1 ml of $1 \times$ PBS was added. The vial was vortexed for 2 min and then sonicated for 5 min in a sonication bath (Elmasonic) before being vortexed for another 2 min. The supernatant was serially diluted, and CFU of wild types and surfactant mutants were enumerated by growing the strains on LB agar containing appropriate antibiotics to select for either the spontaneous streptomycin-resistant wild-type strain or the kanamycin-resistant mutants.

Hydrocarbon utilization assay. To measure the ability of wild types and surfactant knockout mutants to grow on diesel or dodecane as the sole source of carbon, Bushnell-Haas broth (0.2 g liter^{-1} MgSO_4 , $0.02 \text{ g liter}^{-1}$ CaCl_2 , 1.0 g liter^{-1} KH_2PO_4 , 1.0 g liter^{-1} K_2HPO_4 , 1.0 g liter^{-1} NH_4NO_3 , and $0.05 \text{ g liter}^{-1}$ FeCl_3 ; pH 7.2) was supplemented with 1% diesel (commercial diesel, locally sourced) or 1% dodecane (for synthesis; Merck) (16). Bushnell-Haas broth without an additional carbon source was used as a negative control. In control experiments, to complement surfactant knockout mutants, between 0.23 and 0.265 mg ml^{-1} of isolated wild-type (WT) surfactants or 0.1 mg ml^{-1} Tween 20 was used as a supplement. Bacteria were grown overnight in LB, diluted $100 \times$ using Bushnell-Haas broth without a carbon source. The diluted bacterial suspensions were inoculated into 50-ml broth cultures in 250-ml Erlenmeyer flasks. Cultures were incubated at 30°C and 200 rpm for up to 17 days. Cell density was regularly measured by determining the optical density at 600 nm using a spectrophotometer (Biochrom WPA CO8000; Biowave). All experiments were performed in three biological replicates.

Statistical analysis. Statistical analysis was performed using Prism 9 (GraphPad). To analyze growth data in liquid culture, two-way analyses of variance (ANOVA) with Tukey's multiple-comparison test were performed. To analyze wild-type and corresponding knockout mutant growth *in planta*, two-way ANOVA with Šidák's multiple-comparison test was performed.

Data availability. Newly determined 16S rRNA gene sequence data have been deposited in the NCBI database under accession numbers [MW603784](https://doi.org/10.1101/2023.05.11.541111) to [MW603787](https://doi.org/10.1101/2023.05.11.541112).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

We thank Adrien Burch and Steven Lindow, UC Berkeley, for the kind gift of the *Pseudomonas* strains. We thank Paula Jameson for critically reading the manuscript and Thomas Evans for technical support.

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