The influence of resource overlap on bacterial reproductive success in the phyllosphere

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Abstract

The phyllosphere is colonized by rich microbial communities, despite sparse and heterogeneously distributed resources. This resource limitation is expected to drive bacterial competition, resulting in either exclusion or coexistence based on the fitness differences and resource overlap between species. Here, we investigated the impact of competition in bacterial colonization and growth of the epiphyte *Pantoea eucalypti* 299R (*Pe*299R). To that end, pairwise competition experiments between *Pe*299R and diverse phyllosphere-colonizing bacteria were performed *in vitro* and in the *Arabidopsis thaliana* phyllosphere. Resource overlap was determined as the similarity in resource utilization *in vitro*. We found an effect of both resource overlap and phylogenetic relationships in the competition outcome between *Pe*299R and individual competitors *in vitro*. To account for bacterial individuality in the phyllosphere, we employed a single cell bioreporter to determine the number of divisions that individual cells from *Pe*299R populations underwent when challenged with individual bacterial competitors. We observed that at the single-cell level, resource utilization similarities and phylogenetic relationships were weakly correlated with *Pe*299R reproductive success. We observed contrasting results for two strains (*Arthrobacter* sp. Leaf145 and *Sphingomonas melonis* Fr1) that, despite sharing either a high or low resource overlap with *Pe*299R, both negatively affected the reproductive success of *Pe*299R. Interestingly, we also observed facilitative effects of *Methylobacterium* sp. Leaf85. This work furthers the understanding of bacterial assembly processes in heterogenous environments. Our high-resolution observations are important to build an ecological framework to predict competition outcomes in the phyllosphere and to design future bacterial biocontrol applications.

Keywords: Competition, Bacterial fitness, Leaf surface, Epiphytes, Community assembly, Single cells, Plant-microbe interactions, CUSPER

Significance Statement

For plant and consumer protection, plant microbiota manipulation has recently garnered much attention. To manipulate the plant microbiota while acknowledging its ecological context, understanding the mechanisms of microbial community assembly in the phyllosphere is essential. We explored the effect of resource overlap between competing species on the fitness of a focal species at the single-cell resolution using a bottom-up approach. Using a combination of *in vitro* and *in planta* pairwise co-inoculation experiments, we found that competition outcomes are associated with resource overlap and phylogenetic relationships. Although this effect is not strong enough to promote competitive exclusion, we provide examples of competitors that affect the growth of a focal species depending on their resource overlap.

Introduction

For bacteria, the phyllosphere —mostly represented by leaf surfaces— constitutes a challenging environment where resources are limited and heterogeneously distributed (1, 2). This fragmented microbial habitat promotes cell-to-cell contact and should directly impact on community dynamics by short-distance interactions (1, 3–5), leading to the local competition for resources and spatial structuring of bacterial communities (6–8). Resource competition results in either exclusion or coexistence depending on the strength of interspecies interactions in relation to conspecific interactions, that is, interactions within a species (9). Coexistence maintains community diversity and is promoted when competition against a different species is weaker than competing with conspecifics. The strength of interspecific competition can be alleviated by mechanisms that increase niche differences, such as niche overlap (10).

Due to the environmental heterogeneity of the phyllosphere, 'coarse-grained' investigations, such as those considering only a whole leaf or plant as unit of investigation, are of limited suitability when studying local interactions of leaf-associated bacteria. Therefore, to better understand bacterial growth dynamics in the phyllosphere, individual cells must be the subject of investigation, as every cell may experience a different fate (5). A bioreporter used to estimate the number of divisions of individual cells, CUSPER ("repsuc" read backwards, from "reproductive success"), has shown that bacteria experience high variations of reproductive success in the phyllosphere (11, 12). Due to the heterogeneously distributed and limited resources on leaves, the reproductive success of a bacterial cell depends on the local habitability. Consequently, it has been shown that leaves that were pre-colonized with a conspecific strain reduced the reproductive success of CUSPER bioreporter cells proportional to the pre-colonizer density (13). However, interspecific resource competition at the single-cell resolution in the phyllosphere has not been explored.

Here, we tested if resource competition *in vitro* correlates with reproductive success *in planta*. We expect that strong negative impacts *in vitro* will also strongly reduce single cell reproductive success in the phyllosphere. To that end, the epiphyte *Pantoea eucalypti* 299R (*Pe*299R) was challenged with a range of diverse phyllosphere-colonizing bacteria in pairwise competition experiments. Similarities in resource utilization profiles *in vitro* were used as a proxy to estimate resource overlap and to select potential strong and weak competitors of *Pe*299R. Finally, bacterial fitness in the phyllosphere was evaluated using the CUSPER bioreporter in the presence of competitors with different degrees of resource overlap to *Pe*299R.

Results

Bacterial Resource Utilization Profiles. A resource utilization profile for a group of phyllosphere-associated bacterial strains (Table 1, *Appendix SI*, Table S1) was determined by growing each strain in defined minimal media (MM) supplemented with either 0.2% w/v glucose, fructose, malate, sorbitol, or methanol, and comparing the biomass yields across these different growth conditions. Carbon sources were selected based on the detection and abundance of metabolites extracted from whole arabidopsis leaves (14). Additionally, methanol was chosen as it is released from leaves (15, 16). Hierarchical clustering showed four clades based on the growth of the studied strains in the amended minimal medium (Fig. 1*A*). The focal species *Pe*299R grew on glucose, fructose, and malate, but not on either sorbitol or methanol. The clustering grouped *Pe*299R closely with the gammaproteobacterium *Pseudomonas koreensis* P19E3 (*Pk*P19E3) and the actinobacterium *Arthrobacter* sp. Leaf145 (*Arthr*L145) which both showed similar growth in glucose, fructose, and malate. To a lesser extent, *Pe*299R grouped with members of *Alpha*-, *Beta*-, and other *Gammaproteobacteria*, as well as the actinobacterium *Aeromicrobium* sp. Leaf245 (*Aerom*L245). The alphaproteobacterium *Methylobacterium* sp. Leaf85 (*Methy*L85), *Methylorubrum* sp. Leaf92 (*Methy*L92), and *Bradyrhizobium* sp. Leaf396 (*Brady*L396) grew most efficiently in malate compared to the other resources. A fourth clade was defined by strains with the lowest overall biomass yield in the tested growth conditions, including *Sphingomonas* sp. Leaf357 (*Sphin*L357) and *Rhodococcus* sp. Leaf225 (*Rhodo*L225) with a comparatively lower growth than

Table 1. Strains used in this study.

Genus species	Strain	Abbreviation
Acidovorax sp.	Leaf84	*Acido*L84
Aeromicrobium sp.	Leaf245	*Aerom*L245
Agreia sp.	Leaf335	*Agrei*L335
Arthrobacter sp.	Leaf145	*Arthr*L145
Bradyrhizobium sp.	Leaf396	*Brady*L396
Methylobacterium sp.	Leaf85	*Methy*L85
Methylorubrum sp.	Leaf92	*Methy*L92
Microbacterium sp.	Leaf320	*Micro*L320
Pseudomonas koreensis	P19E3	*Pk*P19E3
Pseudomonas syringae	B728a	*Pss*B728a
Rhodococcus sp.	Leaf225	*Rhodo*L225
Sphingomonas melonis	FR1	*Sm*FR1
Sphingomonas phyllosphaerae	FA2	*Sp*FA2
Sphingomonas sp.	Leaf17	*Sphin*L17
Sphingomonas sp.	Leaf357	*Sphin*L357
Pantoea eucalypti	299R	*Pe*299R

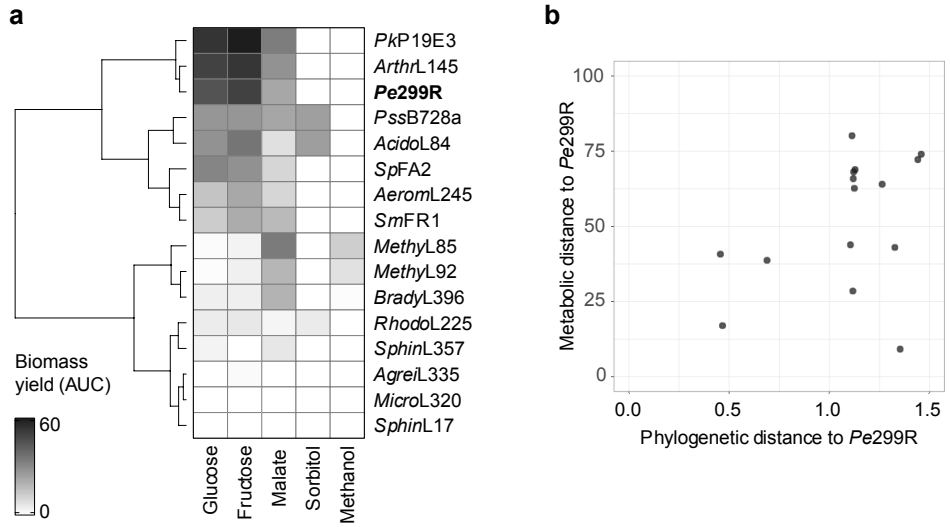

Figure 1. Resource utilization profile of phyllosphere-associated bacteria. (*A*) Species resource utilization matrix. Biomass yield is depicted as the area under the growth curve (AUC) from OD_{600} measurements. Focal species *Pe*299R is highlighted in bold. Hierarchical clustering was based on similarities in resource utilization profiles between species. (*B*) Relationship between phylogenetic distance (substitutions per site) and metabolic distance (Euclidean distance from *A*) of potential competitor species and *Pantoea eucalypti* 299R (*Pe*299R). Refer to Table 1 for the full name of each phyllosphere-associated strain.

strains in the other clades for glucose, fructose, malate, and sorbitol, while *Agreia* sp. Leaf335 (*Agrei*L335), *Microbacterium* sp. Leaf320 (*Micro*L320), and *Sphingomonas* sp. Leaf17 (*Sphin*L17) failed to grow in every growth condition. To test whether metabolic distance correlates with phylogenetic distance between the tested strains and *Pe*299R, metabolic distances were calculated as the Euclidean distances between the resource profiles of *Pe*299R and a second species. Phylogenetic distances were calculated as the distance between branches in a reconstructed phylogenetic tree (*Appendix SI*, Fig. S1). A weak correlation was found between phylogenetic and metabolic distance (Fig. 1*B*, *p*-value = 0.123, adjusted R^2 = 0.109, Pearson's correlation = 0.416), suggesting that phylogeny does not explain metabolic differences between *Pe*299R and the rest of the selected phyllosphere-associated bacteria.

Resource Competition *In Vitro.* The ability of a competitor to affect the growth of a fluorescently red-labeled *Pe*299R (*Pe*299R::mSc, *Appendix SI*, Table S1) was evaluated *in vitro*. The growth of each strain was measured in MM supplemented with multiple resources ($MM_{5\times C}$, 0.125% w/v of a mix of glucose, fructose, malate, sorbitol, and methanol) to compare growth yields of potential competitors of *Pe*299R::mSc. The resource concentration was chosen based on the highest fluorescence readout without compromising growth of *Pe*299R at the lower range of resource concentration (*Appendix SI*, Fig. S2). In general, most competitor bacteria were able to grow on $MM_{5\times C}$, except for *Micro*L320 (Fig. 2, *Appendix* SI, Fig. S3). Strains that yielded growth comparable with *Pe*299R were *Pk*P19E3, *Arthr*L145, *Pseudomonas syringae* pv. *syring ae* B728a (*Pss*B728a), *Sphingomonas phyllosphaerae* FA2 (*Sp*FA2), and *Acidovorax* sp. Leaf84 (*Acido*L84).

At least two strains of each clade from the resource utilization profile that also grew on the $MM_{5\times C}$ growth condition were selected to test their competitiveness against *Pe*299R *in vitro*. To test the effect of a strain on *Pe*299R growth, *Pe*299R::mSc was mixed in equal ratios with a single phyllosphere-associated strain and fluorescence intensity was measured over time as a proxy for *Pe*299R::mSc biomass (17). Then, *Pe*299R::mSc growth yields in competition with an interspecific competitor was normalized by the effect of *Pe*299R::mSc in intraspecific competition (i.e., *Pe*299R::mSc mixed with its non-tagged parental strain). In these conditions, the presence of *Pk*P19E3, *Pss*B728a, or *Arthr*L145 negatively impacted the growth of *Pe*299R::mSc, while the other tested strains exhibited a lower impact on the growth of *Pe*299R::mSc (Fig. 3*A*). Based on the maximal fluorescence intensity of the *Pe*299R::mSc as monoculture, the presence of other strains did not lead to increased growth of *Pe*299R::mSc, suggesting that all strains were true competitors. Additionally, no competitor exhibited direct inhibitory effects on *Pe*299R (*SI Appendix,* Fig. S4), suggesting that resource competition rather than interference competition (e.g., antibiosis) is driving species interactions between the tested bacteria and *Pe*299R.

To determine the relationship between resource overlap, phylogenetic relationships, and competitiveness *in vitro*, a metabolic distance was estimated based on the similarity of resource utilization profile between competitors and *Pe*299R::mSc. Both metabolic and phylogenetic distance between a competitor and *Pe*299R::mSc were used as explanatory variables to evaluate their effect on the ability to affect *Pe*299R::mSc growth. Regression analysis found an interaction between metabolic and phylogenetic

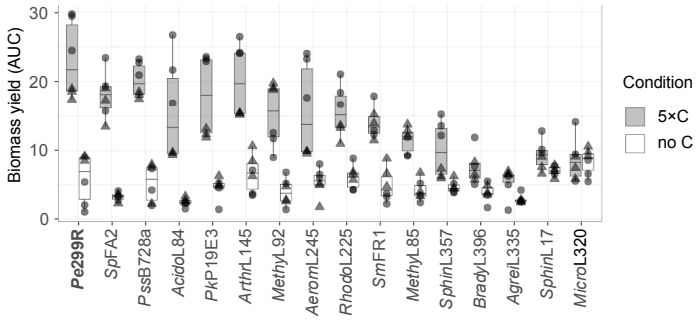

Figure 2. Growth of phyllosphere-associated bacteria in multiple carbon sources. Growth of phyllosphere-associated bacteria in minimal media supplemented with 0.125% w/v of mixed carbon sources ($MM_{5\times C}$). Focal species *Pe*299R is highlighted in bold. Biomass yield was determined as the area under the growth curve (AUC) from OD_{600} measurements of individual species grown in a mix of five carbon sources (5×C: glucose, fructose, malate, sorbitol, methanol). As negative control, MM without carbon sources was used. Different shapes indicate results from independent experiments.

distance (Figure 3*B*, $p\text{-value}_{interaction} < 0.05$, adjusted R^2 = 0.80), suggesting that the response of a competitor on *Pe*299R growth depends on both metabolic and phylogenetic differences. In summary, both resource overlap and phylogenetic relatedness have a role in the competitiveness of a bacterial strain in affecting *Pe*299R::mSc fitness *in vitro*.

***Pe*299R Single-Cell Reproductive Success in Competition.** Here, an improved version of the CUSPER bioreporter plasmid was constructed (*SI Appendix*, Fig. S5) and was used to develop *Pe*299R::Tn*7*::Gm^R::mSc (pProbe_CUSPER) –hereafter referred to as $Pe299R_{CUSPER}$–. $Pe299R_{CUSPER}$ is a bioreporter that estimates the reproductive success (RS) of immigrant cells in a new environment by back-calculating the number of divisions a cell underwent since its arrival (11–13). Reproductive success was determined by measuring the decrease in single cell fluorescence compared to the mean fluorescence of the population at time zero (t_0). Under optimal homogeneous conditions, it is expected that the entire population will divide until the lower limit of detection for the GFP signal is reached. By contrast, factors such as environmental heterogeneity and presence of competitors are expected to decrease the success of the immigrant population. The newly developed $Pe299R_{CUSPER}$ was evaluated in both liquid culture and on arabidopsis leaves. As expected, the $Pe299R_{CUSPER}$ population increased over time in both environments (PERMANOVA, R^2 = 67.5%, *p*-value = 0.001). In liquid culture, the cell population divided at regular intervals (~ 1 h), suggesting that the reproductive success of individual cells within a population was normally distributed (*SI Appendix,* Figure S6*A*). By contrast, on arabidopsis leaves, subpopulations differentiated from each other over time, resulting in a non-normally distributed $Pe299R_{CUSPER}$ cell population after 36 h (*SI Appendix,* Fig. S6*B*). In both cases, the limit of detection was approximately five divisions before the fluorescence of cells was not significantly different from the background (*SI Appendix,* Fig. S7).

The effect of a competitor on the reproductive success of $Pe299R_{CUSPER}$ was evaluated in the arabidopsis phyllosphere. A set of seven phyllosphere-associated bacteria: *Pk*P19E3, *Pss*B728a, *Arthr*L145, *Pe*299R, *Methy*L85, *Sm*FR1, and *RhodoL*225, were selected based on their ability to affect *Pe*299R

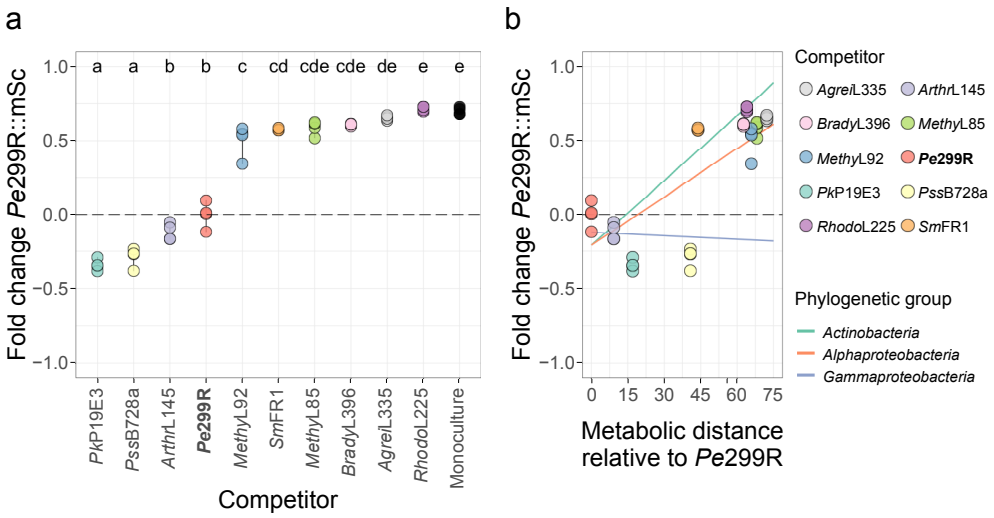

Figure 3. Fitness of *P. eucalypti* 299R in competition with phyllosphere-associated bacteria in liquid culture. (*A*) Ranked fold change in fluorescence of *P. eucalypti* 299R::Tn7::mSc::Gm^R (*Pe*299R::mSc) in interspecific competition relative to intraspecific competition (*Pe*299R, bold) in $MM_{5\times C}$. Differences between groups were tested with ANOVA (α = 0.05) and letters display multiple pairwise comparisons using Tukey's post hoc test with Bonferroni correction. (*B*) Relationship between metabolic distance between *Pe*299R::mSc and a second species on *Pe*299R fitness. Continuous colored line indicates fitted model at different degrees of phylogenetic relatedness between a competitor (green: *Actinobacteria*, orange: *Alphaproteobacteria*, purple: *Gammaproteobacteria*) and *Pe*299R::mSc (*Gammaproteobacteria*), based on the regression model $y = -0.12 - 0.057\ X_{phylogeny} - 0.00755\ X_{metabolism} + 0.016094\ X_{phylogeny}\ X_{metabolism}$. In (*A*) and (*B*), a horizontal dashed line indicates no fold change (y = 0) with respect to intraspecific competition as a reference.

growth *in vitro*. Four-week-old arabidopsis plants were co-inoculated with *Pe*299R$_{CUSPER}$ and the respective competitors to estimate single-cell reproductive success *in planta* as well as the changes in population densities. As expected, we first observed that in all conditions, *Pe*299R$_{CUSPER}$ differentiated into subpopulations over time with different degrees of reproductive success (Fig. 4). *Pe*299R$_{CUSPER}$ cells have divided with a median between four to five times at 24 and 36 h (median$_{RS}$ = 3.98—5.11). However, the presence of competitor species influenced the distribution of *Pe*299R$_{CUSPER}$ populations during leaf colonization (Kruskal-Wallis, $p < 0.0001$). Particularly, the presence of the competitors *Arthr*L145, *Pss*B728a, *Rhodo*L225, and *Sm*FR1 decreased the reproductive success of *Pe*299R$_{CUSPER}$ at 24 h (Fig. 4). This effect was maintained over time when *Arthr*L145 or *Sm*FR1 was present, but only transient in the presence of *Rhodo*L225, as no differences were observed at 36 h for this competitor (Fig. 4). By contrast, *Pk*P19E3, *Pss*B728a, and *Methy*L85 showed to have a positive effect on the reproductive success of *Pe*299R$_{CUSPER}$ at 36 h (Fig. 4). We also observed that when *Pe*299R or *Arthr*L145 were competitors, the median RS of *Pe*299R$_{CUSPER}$ decreased from 24 to 36 h (Dunn's test, $p < 0.05$). The generalized linear mixed model with the best fit (*SI Appendix*, Table S2, pseudo-R^2 = 0.043) showed that the observed reproductive success in the realized *Pe*299R$_{CUSPER}$ population was associated with the interaction between metabolic and phylogenetic differences (GLMM, $p < 0.05$), and the interaction between metabolic differences and time of sampling (GLMM, $p = 0.016$). These results suggest a significant but rather small effect of a competitor species in the reproductive success of *Pe*299R based on their phylogenetic relationships and resource overlaps in the phyllosphere.

We found that the presence of a competitor also affected *Pe*299R$_{CUSPER}$ over time at the population-level (two-way ANOVA, $F = 2.76$, $p = 0.0025$ for the interaction between the presence of competitor and time of sampling). However, single-cell and population-level estimations of the fitness effect of *Pe*299R$_{CUSPER}$ in competition in the phyllosphere were partially consistent. For example, only the presence of *Sm*FR1 negatively affected the density of the whole *Pe*299R$_{CUSPER}$ population, while *Methy*L85 and *Arthr*L145 positively affected the focal strain at 36 h (Fig. 5). The effect of other strains was not statistically different to the population density of *Pe*299R$_{CUSPER}$ in intraspecific competition (Fig. 5). Multiple regression analysis suggested that population-level density of *Pe*299R in competition is associated with an interaction between metabolic differences an phylogenetic relationships with its competitors, as well as the time of sampling (Linear mixed-effects model, $\beta_{interaction}$ = -0.031, marginal R^2 = 0.54, conditional R^2 = 0.92, $p = 0.0008$).

The distribution of immigrant cells that experienced different levels of reproductive success was analyzed as relative fractions of the initial population. Subpopulations were assigned based on the estimated reproductive success of an arrival cell, that is, the number of cells that divided from 0 to >5 times after inoculation (*SI* Appendix, Table S3). The fraction of each *Pe*299R$_{CUSPER}$ subpopulation was then used to compare the differences in distribution of *Pe*299R$_{CUSPER}$ when a competitor was present in the phyllosphere. We found that the sampling time explained most of the variation in the composition of the *Pe*299R$_{CUSPER}$ population (PERMANOVA, R^2 = 74%, $p = 0.001$) rather that the presence of a competitor (PERMANOVA, R^2 = 2.3%, $p = 0.217$). However, we noticed that the subpopulation of *Pe*299R$_{CUSPER}$ cells that divided five times was larger and contributed mostly to the final population when *Pk*P19E3, *Pss*B728a, and *Methy*L85 were present at 36 h, in comparison with *Pe*299R$_{CUSPER}$ in intraspecific competition (Fig. 6, *SI* Appendix, Fig. S8). However, these observations were not linked to either phylogeny (linear regression, $p = 0.69$) or the metabolic differences estimated *in vitro* (linear regression, $p = 0.99$).

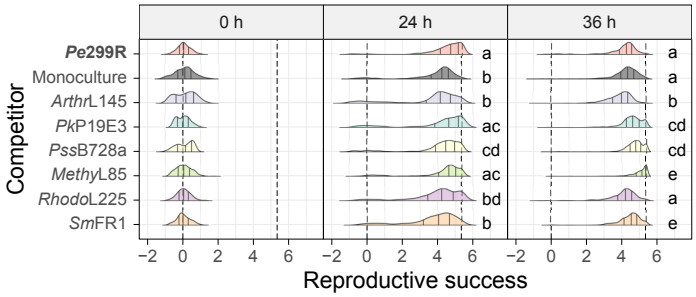

Figure 4. Single-cell reproductive success of *Pe*299R$_{CUSPER}$ populations in competition in the phyllosphere. Distribution of the reproductive success of single *Pe*299R$_{CUSPER}$ cells in the presence of a competitor or as monoculture at 0, 24, and 36 h post-inoculation onto arabidopsis leaves. Intraspecific competition (*Pe*299R) is highlighted in bold. Each violin plot indicates the median and the interquartile range. Groups within each time point were compared using Kruskal-Wallis *H* test by ranks, with a significance level of α = 0.0001. Letters display multiple pairwise comparisons between groups within each time point using Dunnett's tests with Bonferroni correction.

The relative increase in *Pe*299R$_{CUSPER}$ population from the initial inoculum at a given time point can be estimated based on the fraction of cells in a particular subpopulation and the number of divisions that a cell is expected to undergo upon arrival in the phyllosphere (12). This increase estimated by single-cell measurements was highly correlated with the increase in population size at the CFU level (*SI Appendix*, Fig. S9, R^2 = 0.79). However, the increase in *Pe*299R$_{CUSPER}$ population was associated with the sampling time (two-way ANOVA, $p < 0.05$) but not the presence of a competitor (two-way ANOVA, $p = 0.36$) (*SI Appendix*, Fig. S10). This pattern was not explained by the phylogenetic relationships (linear regression, $p = 0.48$) or the metabolic differences estimated *in vitro* (linear regression, $p = 0.61$) between *Pe*299R and a competitor, but only by sampling time (linear regression, $p = 0.023$). Our findings suggest that competition *in vitro* and resource overlap are not the best predictors of bacterial population increase in the phyllosphere.

Discussion

In this study, the relationship between resource competition and reproductive success in the phyllosphere was investigated. The competitive ability of a taxonomically diverse group of phyllosphere-associated bacterial species were estimated *in vitro* and used to rank their competitiveness against *Pe*299R in both liquid culture and the arabidopsis phyllosphere. *Pe*299R was selected as a focal species as it is a model organism that has been widely used to understand bacterial physiology and ecology in the phyllosphere (2, 7, 18–21). However, its interactions with other species in this environment has been underexplored. Lindow and Wilson (1994) showed a negative correlation between coexistence and similarity in resource utilization for a number of pairs of phyllosphere-associated bacteria, including *Pe*299R (22). Here, a wide range of bacterial species, including the understudied Gram-positive phyllosphere-associated bacterial phylum *Actinobacteria*, were included to understand whether similarity in resource use had a phylogenetic signal. Our results show that the clustering of species based on their resource utilization profiles did not correlate with their phylogenetic relationships. Particularly, *Pe*299R grouped closely with the gammaproteobacterium *Pk*P19E3 and the actinobacterium *Arthr*L145. This is in line with the findings that carbon utilization is a trait that is widespread and not phylogenetically conserved in *Bacteria* (23).

Potential competitors were selected according to their similarity in resource use with *Pe*299R, as competitive exclusion is expected to be favored under high metabolic overlap in conditions where resources are shared (24). Among these competitors, *Pk*P19E3 and *Arthr*L145 were of special interest due to their high metabolic similarity, but different degrees of phylogenetic relationships with *Pe*299R. Other groups included representative members of the phyllosphere microbiota, such as the highly abundant *Sphingomonas*, *Methylobacterium*, and *Pseudomonas* spp. (25), and less abundant members, such as *Bradyrhizobium*, *Agreia,* and *Rhodococcus* spp. (26). The impact of competitor species on the fitness of *Pe*299R *in vitro* was most likely a consequence of resource competition, as no direct antagonistic effects were found for the tested bacteria on *Pe*299R in a double layer assay (*SI Appendix,*

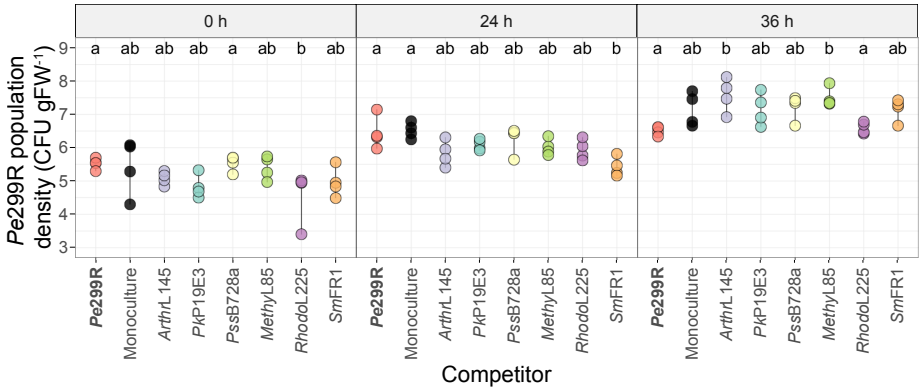

Figure 5. *P. eucalypti* 299R population density in competition in the phyllosphere. Population size of *P. eucalypti* 299R::Tn7::mSc::Gm^R (pProbe_CUSPER) (*Pe*299R$_{CUSPER}$) on arabidopsis plants at 0, 24, and 36 h. *Pe*299R$_{CUSPER}$ was co-inoculated with a near-isogenic strain *Pe*299R, or with a different phyllosphere-associated bacteria in equal ratios. *Pe*299R$_{CUSPER}$ as monoculture was included in the analysis. Intraspecific competition (*Pe*299R) is highlighted in bold. Each data point represents the CFU of *Pe*299R per gram of fresh leaf weight (CFU gFW^{-1}) of independent plants (n = 4). Groups were compared using two-way ANOVA, with a significance level of α = 0.05. Letters display multiple pairwise comparisons between groups within each time point using Tukey's post hoc test with Bonferroni correction.

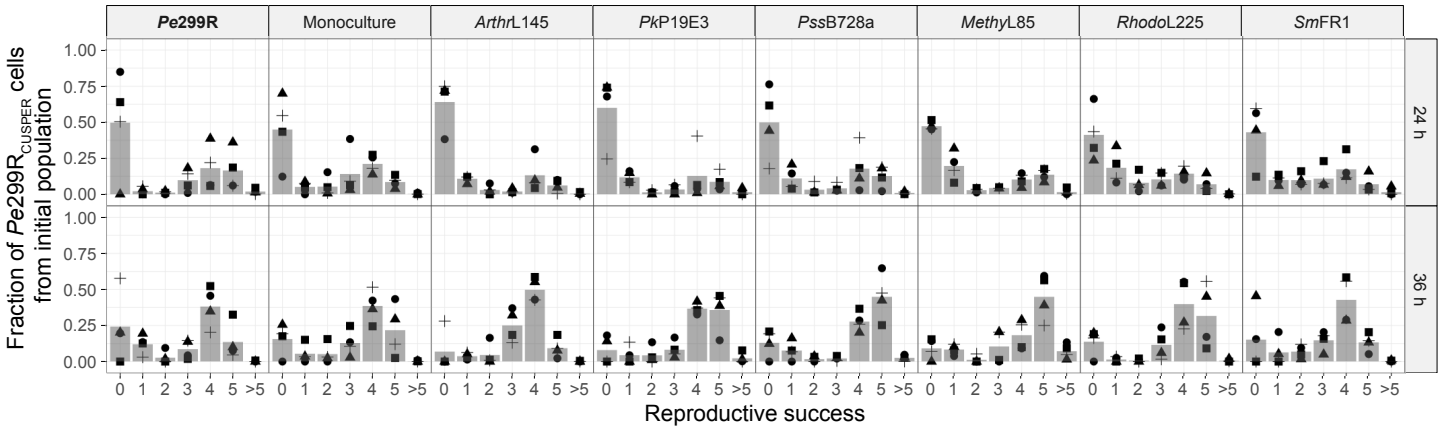

Figure 6. Relative fractions of *Pe*299R$_{CUSPER}$ subpopulations based on their relative contribution to the *t*0 or founder population after 24 and 36 h in presence of different competitors. Each data point represents a fraction of the subpopulations per independent biological replicate (symbols) and the bar represents the mean relative fraction for all replicates. Intraspecific competition (*Pe*299R) is highlighted in bold.

Fig. S4). This is not surprising since interference competition through antibiosis is relatively rare between phyllosphere-associated bacteria, as fewer than 2% of binary interactions resulted in growth inhibition (27). Furthermore, inhibitions are mostly driven by only two bacterial orders, the *Pseudomonadales* and *Bacillales*, and the only candidates belonging to *Pseudomonadales* (*Pk*P19E3 and *Pss*B728a) did not inhibit *Pe*299R.

The epiphytes *Pk*P19E3 and *Arthr*L145, as well as *Pss*B728a, showed a strong negative effect on *Pe*299R fitness *in vitro*. In contrast, competitors with low resource similarity (e.g., *Sm*FR1, *Methy*L85, *Methy*L92, *Brady*L396, *Agrei*L335, and *Rhodo*L225) had a minor impact on *Pe*299R fitness, suggesting a strong relation between metabolic similarities and *Pe*299R fitness *in vitro*. A regression model showed an interaction effect of phylogenetic and metabolic differences on the fitness of *Pe*299R when in competition for multiple resources. These results suggest that under high resource overlap, phylogeny has a small effect on the growth of *Pe*299R. However, at lower resource overlap, distantly related species had a smaller impact on *Pe*299R fitness. A recent study on the interactions between and within *Pseudomonadales* and *Enterobacterales* showed that interactions between species have both a phylogenetic and metabolic signal *in vitro* (28), where negative interactions are more common between closely-related species, which also happened to have a higher level of resource overlap. Overall, the findings of the *in vitro* observations indicate that similarities in resource usage and phylogenetic relationships explain the strength of pairwise competitive interactions.

The redeveloped bioreporter *Pe*299R$_{CUSPER}$ was used to estimate the single-cell reproductive success of *Pe*299R in competition with selected phyllosphere-associated strains *in planta*, which were shown to affect *Pe*299R fitness *in vitro* to varying degrees. This bioreporter relies on the fluorescence intensity of individual cells, which can be traced back to estimate the number of divisions a cell underwent based on the dilution of a fluorescent protein after cell division (11). This bioreporter was instrumental in that bacterial populations in the phyllosphere separate into subpopulations over time (11). In contrast to the initial CUSPER bioreporter, the redeveloped bioreporter produces an additional red fluorescent protein and carries a recently developed green fluorescent protein mClover3 on a plasmid, rather than a single copy of GFPmut3 that was chromosomally inserted. As expected, the results using the redeveloped and enhanced *Pe*299R$_{CUSPER}$ were consistent with the first iteration: growth of *Pe*299R in liquid culture was normally distributed, while growth in the arabidopsis phyllosphere resulted in subpopulations that experienced different numbers of divisions (*SI Appendix*, Fig. S6 and S7). These results suggest that the variable habitability of the phyllosphere is a common feature of plant species, as previous works on CUSPER were performed on bush bean leaves (*Phaseolus vulgaris*). The differences in reproductive success of *Pe*299R subpopulations can be linked to heterogeneous amounts of resources such as carbohydrates and water that immigrant bacterial cells are exposed to upon arrival on the leaf surface (1, 2, 29). After 36 hours, we observed a reduction of cells that did not divide after arriving in the phyllosphere (RS = 0) (Fig. 6, *SI Appendix* Fig. S6B and S8). This is likely the result of dying cells, rather than initially unsuccessful cells migrating to new sites that would allow them to grow, as bacterial relocation has been suggested to happen during early stages of colonization in the phyllosphere (20). Furthermore, there were no signs of dew in our system that was under controlled relative humidity conditions that would allow for migration to happen. However, a fraction of cells with RS = 0 is expected to be true unsuccessful cells that could have undergone cell death and lysed, which cannot be determined with our bioreporter.

Recognizing the variable fate of bacterial cells during leaf colonization, we tested the effect of resource overlap in competitive bacterial interactions at the single-cell level. Particularly, we found that *Arthr*L145 significantly affected the growth of *Pe*299R by restricting the reproductive success of *Pe*299R to a median of four cell divisions. *Arthr*L145 was selected as a strong competitor of *Pe*299R as this strain showed the highest similarity in resource usage, biomass yield in multiple carbon source growth condition, and a negative impact on *Pe*299R fitness *in vitro*. Altogether, *Arthr*L145 was the best example of resource overlap being a stronger predictor of competition outcome than phylogeny. Only recently, *Arthr*L145 was also shown to have a protective effect in arabidopsis against the foliar pathogen *Pseudomonas syringae*

pv. *tomato* DC3000 (*Pst*DC3000) through a mechanism independent of the BAK1/BKK1-associated plant immune response (30), suggesting that microbe-microbe interactions such as resource overlap, could be driving the reduction in *Pe*299R populations as well.

*Pk*P19E3 and *Pss*B728a were also selected as a strong competitor because of its similarity in resource usage and phylogenetic relationships, and because they had a strong negative effect on *Pe*299R in competition *in vitro*. Unexpectedly, these strains positively affected the growth of more successful *Pe*299R subpopulations, showing an increase in relative abundance of cells that divided five times or more (Fig. 6). This suggests that mechanisms other than resource competition were influencing the interactions between *Pe*299R and *Pk*P19E3 or *Pss*B728a. *Pseudomonas* spp. are recognised for producing biosurfactants, which are amphiphilic compounds that improve the permeability of resources onto the leaf surface and increase bacterial survival due to their water retaining hygroscopic nature (31–33). By modifying their local environment, *Pseudomonas* spp. could indirectly benefit *Pe*299R. Alternatively, these strains could engage in cooperative interactions such as cross-feeding, as it was recently shown that potential between *Pantoea* sp. and *Pseudomonas koreensis* in the *Flaveria robusta* leaf apoplast (34). However, further investigations are required to understand the mechanisms that result in beneficial interactions in the phyllosphere.

We tested whether competition can be influenced by resource overlap among phyllosphere-associated bacteria. While high resource overlap was expected to have a negative impact on *Pe*299R growth, we also expected that low resource overlap would result in neutral or positive effects on *Pe*299R. Indeed, we found that the presence of *Methy*L85 consistently supported *Pe*299R growth in the phyllosphere, both at the single-cell and CFU level. *Methy*L85 belongs to a group of resource specialists and facultative methylotrophs from the genus *Methylobacterium*. Methanol utilization is a fitness advantage in the phyllosphere, as methylotrophs can utilize the released methanol from the plant cell wall metabolism (35). As one carbon metabolism is highly overrepresented in proteomes of methylobacteria on leaves (25, 36), it is expected that *Methy*L85 utilizes methanol as a carbon source and does not compete with *Pe*299R for their preferred resources. Possibly, additional biomass and surfactant production may act as a water retaining factor which increases survival and spread of bacteria (31, 32, 37).

In other cases, strains such as *Sm*FR1 showed a transient negative effect on the growth rate of *Pe*299R subpopulations in the phyllosphere, despite displaying a low resource usage similarity and low impact on *Pe*299R fitness *in vitro*. The poor relationship between these factors and the ability of some strains to affect *Pe*299R reproductive success could be due to indirect effects, such as through modulation of the environment via host-microbe interactions. *Sm*FR1 has been previously shown to decrease the population density of *Pst*DC3000 in arabidopsis, potentially through priming of plant immune defense responses (30, 38, 39). However, the spatio-temporal dynamics of these changes and their significance to other microbial inhabitants remain elusive.

Overall, we observed a relationship between the similarity in resource usage between pairs of species and single-cell reproductive success. It has been shown that similarity in resource usage is negatively correlated with the level of coexistence between pairs of epiphytic bacteria (22, 40). Our findings support the hypothesis that resource competition plays a role in community assembly processes in the phyllosphere. The observed relationship suggests that the similarity in resource usage profiles impact to some extent on the reproductive success of a population in interspecific competition compared to intraspecific competition. This relationship was stronger and more predictive in a homogeneous environment than in the phyllosphere. These differences resulted in a relative increase of cells that were less successful when competitors with similar resource profiles were present. However, competition did not drive the exclusion of any species in any of the tested pairwise interactions, suggesting that the strength of competition is alleviated by either fitness differences or stabilizing mechanisms, enabling the coexistence of the studied species in the phyllosphere. These findings establish a link between resource overlap and reproductive success, supporting the notion that resource competition is one of many factors that impacts on community assembly processes in a spatial and temporal manner in heterogeneous environments such as the phyllosphere.

Understanding the impact of resource competition in bacterial community assemblage in the phyllosphere has major implications in developing effective biocontrol strategies against biotic stresses. Many bacterial foliar pathogens undergo an epiphytic lifestyle that is characterized by population growth before colonizing the plant host endophytic compartments (41). The rational design of biocontrol agents or communities that effectively reduce pathogen populations in the phyllosphere through competitive interactions with native phyllosphere-associated microbial residents could prevent crop losses caused by microbial diseases.

Materials and Methods

Bacterial Strains and Growth Conditions. *Pe*299R and representative phyllosphere-associated bacterial strains are listed in Table 1 (*Appendix SI*, Table S1). *Pe*299R was used to construct the constitutively red-fluorescent protein producing strain *Pe*299R::mSc and the CUSPER bioreporter strain *Pe*299R$_{CUSPER}$ (*Appendix SI*, Materials and Methods). Bacteria were routinely grown in Reasoner's 2a agar or broth (R2A, HiMedia) at 30°C. Minimal media was used to evaluate growth and competition for defined carbon sources, as described in (17). Carbon sources used were glucose, fructose, sorbitol, malate, and methanol.

Carbon Utilization Profiles. Each strain was grown at 30°C in R2A broth until the late stationary phase. Cells were then harvested by centrifugation at 2,000 × *g* for 5 min, washed twice in PBS, and resuspended in MM to an optical density (OD_{600}) of 0.5. Afterwards, 20 µL of bacterial suspension were added to 180 µL of MM supplemented with 0.2% w/v, or 0.2% v/v for methanol, of a carbon source in flat bottom 96-well microtiter plates (Costar) with four technical replicates per condition. Minimal medium without added carbon source was used as a negative control. Optical density was measured in a FLUOstar Omega microplate reader (BMG Labtech) for 5 days in 24 h intervals. The experiments were conducted twice independently.

Competition for Carbon Sources. Competition assays were performed in MM supplemented with a mix of glucose, fructose, sorbitol, malate, and methanol ($MM_{5\times C}$). Suitable carbon source concentrations were determined as explained in *Appendix SI*, Material and Methods. *Pe*299R::mSc was competed against individual non-fluorescent competitors by mixing both strains in a 1:1 OD_{600} ratio, as described elsewhere (17). Briefly, flat bottom

96-well microtiter plates (Costar) were seeded with 200 µL $MM_{5\times C}$ and a defined mixed bacterial suspension (OD_{600} = 0.05, three technical replicates). *Pe*299R::mSc fluorescence was measured every 5 min for 20 h. Fold change of *Pe*299R::mSc fitness relative to intraspecific competition was calculated as the binary logarithm of the ratio between the AUC of *Pe*299R::mSc in interspecific competition and the mean of the AUC of *Pe*299R::mSc in intraspecific competition.

Evaluation of the *Pe*299R$_{CUSPER}$. The new *Pe*299R$_{CUSPER}$ bioreporter was first evaluated as monoculture in liquid culture and the arabidopsis phyllosphere. A *Pe*299R$_{CUSPER}$ fresh culture was induced with 1 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG) and 50 µg mL^{-1} kanamycin, as described in detail in *Appendix SI*. The IPTG-induced culture was used to either inoculate NB or air sprayed onto arabidopsis plants. Samples were taken at different time points, in which cells were harvested, fixed, and stored at -20 °C until they were analyzed by microscopy.

Plant Inoculation. To determine the fitness effect of *Pe*299R$_{CUSPER}$ in competition with epiphytic bacteria on leaves, arabidopsis plants (*Arabidopsis thaliana* accession Col-0) were grown in Magenta GA-7 tissue-culture boxes containing ½ MS agar media (1.0 % w/v, pH 5.9). Plants were grown in a Conviron A1000 plant growth chamber at 22°C, 80% relative humidity (RH) and short-day photoperiod (11 h light / 13 h dark cycles, light intensity ~120-150 µE m^{-2} s^{-1}). For plant inoculation, bacterial suspensions were adjusted to an OD_{600nm} of 0.05 and mixed in equal ratios for each pair (1:1 competitor:*Pe*299R$_{CUSPER}$). Four-week-old plants were inoculated with 200 µL bacterial suspension per box using a sterile airbrush (KKmoon Airbrush Model T130A). Plants were harvested at 0 and 24 hpi by cutting the complete leaf rosette from the roots using sterile scissors and scalpel and transferring the plant into a 1.7 ml microcentrifuge tube. For each condition, four independent plants were used as biological replicates. After the fresh weight of each plant was determined, 1 mL PBS supplemented with 0.02% Silwet L-77 was added. Samples were shaken in a bead mill homogenizer (Omni Bead Ruptor 24, Omni International) for two cycles of 5 min at a speed of 2.6 m s^{-1} and sonicated for 5 min (Easy 30 H, Elmasonic). Colony-forming units were determined by serial dilutions on R2A agar plates and normalized by the corresponding plant fresh weight (CFU gFW^{-1}), while the remaining supernatants were transferred into a clean 1.7-mL centrifuge tube and centrifuged at 15,000 × *g* for 10 min at 4 °C to collect cells for microscopy. Cells were fixed in 4% paraformaldehyde in 1 × PBS (*Appendix SI*, Materials and Methods).

Microscopy. Fixed bacterial cells recovered from leaves or from liquid culture were mounted on microscopy slides coated with 0.1% w/v gelatine. Images were taken on a Zeiss AxioImager.M1 fluorescent widefield microscope at 1000 × magnification (EC Plan-Neofluar 100×/1.30 Ph3 Oil M27 objective) equipped with Zeiss filter sets 38HE and 43HE (BP 470/40-FT 495-BP 525/50 and BP 550/25-FT 570-BP 605/70, respectively), an Axiocam 506, and the software Zeiss Zen 2.3. At least 100 cells were acquired per biological replicate in three different channels: green (38HE filterset), red (43HE filterset), and phase contrast.

Image Analysis. Images were analyzed using FIJI/ImageJ (version 2.0.0-rc-69/1.52s, (42)). As *Pe*299R$_{CUSPER}$ constitutively expresses mScarlet-I, the red fluorescent channel was used as a mask to select individual cells, using the thresholding method "intermodes" and converted to a binary mask object. Only particles with a size range of 0.5-2.5 µm were selected. Selected objects were manually inspected using the phase contrast images to corroborate the selection of bacterial cells and to delete false positive red fluorescent particles. The mask was then used to determine green fluorescence of *Pe*299R$_{CUSPER}$ cells. In addition, background fluorescence was measured by sampling a random section of background area in each fluorescent image (2).

Estimation of Single-Cell Reproductive Success. The reproductive success of *Pe*299R$_{CUSPER}$ at a time *t* was estimated from background-corrected fluorescence measurements by subtracting the average fluorescence intensity of each cell by the background fluorescence of its corresponding field of view, using the 97.5 percentile point of the background intensity of each replicate (mean + 1.96 × standard deviation). Then, reproductive success of a cell was calculated according to (12). The distribution of reproductive success of the initial cell population was estimated as a fraction of *Pe*299R$_{CUSPER}$ cells from the total population, obtaining a relative subpopulation for each condition (*Appendix SI*, Table S3). The similarity of the resulting distributions was compared by hierarchical clustering using the mean of the relative fraction of *Pe*299R$_{CUSPER}$ subpopulations.

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