

Pseudomonas* intra-genus competition determines protective function of SynComs in *Arabidopsis thaliana

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ABSTRACT

The plant root microbiota is crucial for nutrient acquisition, development, and disease suppression. Although commensal bacteria display host preference, their beneficial impact on their cognate host and mechanisms of species selection by the plant are still unclear. We use bacterial culture collections derived from the two model species *Arabidopsis thaliana* (*At*) and *Lotus japonicus* (*Lj*) to design synthetic communities (SynComs) and test their protective function upon exposure of *At* Col-0 to the detrimental root-colonizing *Pseudomonas* isolate R401. *Lj*-derived SynComs were fully protective, whereas *At*-derived SynComs displayed full protective activity only towards a R401 mutant impaired in the production of inhibitory exometabolites. The protective phenotypes were associated with a reduced titer of the R401 opportunistic pathogen. *In vitro* antagonist assays, *in planta* and *in vitro* bacterial community profiling, as well as strain-swapping and strain-dropout experiments revealed that competition among commensal *Pseudomonas* strains and R401 determines the success of the opportunist, independent of the original host or the phylogeny of the commensals. Furthermore, we determine the carbon utilization potential of these isolates, which may explain the competition with the detrimental strain and the role of host-secreted compounds. Our results provide evidence that intra-genus interactions within SynComs modulate plant health and disease, and that an individual beneficial strain can be sufficient to outcompete an opportunistic relative. This has implications for the successful development of beneficial microbial consortia for agriculture.

Key words: *Pseudomonas*, root microbiota, SynComs, *Arabidopsis*, competition

INTRODUCTION

Plants establish tight relationships with the microorganisms surrounding them. These diverse microorganisms include viruses, bacteria, archaea, fungi, protists, and nematodes, and are collectively referred to as the plant microbiota [1, 2]. The structure of the microbiota is crucial for plant health and protection against pathogens [3, 4], where an imbalance is associated with susceptibility and disease [5, 6]. Plant microbiota diversity and composition is primarily shaped by the environment, i.e., the initial microbial pool in the soil, soil physicochemical properties, geography, and climate [7, 8]. The plant host then functions as a filter [9, 10], especially belowground, where root-secreted compounds such as carbon sources and signaling molecules act as key determinants driving microbial activity and composition [11]. This so-called rhizosphere effect results in a higher microbial density and lower microbial diversity along the soil-root axis. In addition to these main drivers, host species-specific effects on root-associated bacterial microbiota were shown for plants grown in natural environments [12–14], in greenhouse settings [10, 15–19], and in controlled gnotobiotic conditions [17]. Host-specific microbiota composition is likely explained by a signature cocktail of root exudates, a fine-tuned innate immune system, and specific root architecture of individual hosts. Which plant and microbial factors determine these important host-specific communities, and how they are established and maintained remains an active field of research.

The major bacterial taxa in the plant microbiota typically belong to six classes, namely Bacteroidota, Bacillota, Actinomycetota (formerly Bacteroidetes, Firmicutes, Actinobacteria), and alpha-, beta-, and gamma-Proteobacteria [3, 9, 20]. Notably, both plant beneficial and detrimental bacteria are often found within these groups, and even within the same species [21, 22]. Although several microbiota members are detrimental in mono-association experiments under specific laboratory conditions, their detrimental activity is often reduced in a community context, suggesting the existence of mechanisms that suppress disease in nature [23–26]. This can be the result of microbe-microbe interactions, in which antagonism, cooperation, and competition shape community composition [27, 28]. In addition, host physiology and environmental changes will affect these dynamics. Nevertheless, ecological

frameworks predict that diverse communities are more robust against perturbations, which moreover suggests that beneficial microbial traits may only be exerted in a community context [23, 29].

The existence of host species-specific root microbiota and the observation of host preference among commensal bacteria suggest a certain degree of adaptation of the microorganisms to a specific host, i.e., metabolic niche. To what extent the host plant benefits from accommodating distinct yet taxonomically similar strains, e.g., through biotic or abiotic stress alleviation, is unclear.

In this study, we addressed if i) the origin of isolation of bacterial communities affects protection against a detrimental strain, ii) how a detrimental strain impacts community composition *in planta* and *in vitro*, and iii) if microbe-microbe interactions explain the observed phenomena.

We used the previously established bacterial culture collections of isolates from roots of *Arabidopsis thaliana* (At) (At-RSPHERE, [30]) and *Lotus japonicus* (Lj) (Lj-SPHERE, [17]) to design taxonomically diverse synthetic communities (SynComs) and test their potential to provide protection against the opportunistic *Pseudomonas brassicacearum* strain Root401 (R401) in At Col-0 plants [31, 32]. We found that low-complexity SynComs protect against R401 independently of their hosts of isolation, and that SynCom protective function can be abolished by R401 via exometabolite-mediated inhibition of SynCom members. We also revealed that within-genus competition between *Pseudomonas* commensals and the opportunistic *Pseudomonas* R401 explains SynCom protective success. Therefore, we delineate *Pseudomonas* intra-genus competition as a possible strategy to control pathogen emergence in nature.

RESULTS

At-derived and Lj-derived SynComs differentially suppress R401-induced disease in *Arabidopsis*

P. brassicacearum R401 was originally isolated from At Col-0 plants growing in agricultural soil and is part of the At-RSPHERE culture collection [30]. Upon single inoculation on Col-0

seedlings grown in an agar-based system, R401 causes wilting and disease symptoms ([31]; Fig 1A) that were recently shown to be partially dependent on the production of the lipopeptide brassicaeptin A [32]. To test the potential beneficial effect of bacterial communities on plant performance and resistance, we infected sterile Col-0 seedlings and seedlings co-cultivated with two different SynComs of *At*-derived strains (AtSC5 and AtSC6) of diverse taxonomic composition (Materials and Methods, S1 Table) with a dilute culture of R401 (S1 Fig). Inoculation of R401 on *At* Col-0 led to a chlorotic and stunted plant phenotype that was mildly alleviated by AtSC5 and AtSC6, indicating that *At*-derived SynComs largely failed to protect plants from R401 (Fig 1A, B). We also tested two other SynComs derived from the *Lj*-SPHERE collection (LjSC5 and LjSC6) with similar taxonomic structure to AtSC5 and AtSC6 (S1 Table) and found that both rescued the R401-induced plant growth defect to levels of non-infected control plants (Fig 1A, B). The severity of plant wilting and growth impairment was directly linked to R401 colonization capabilities, since bacterial load was generally greater in roots of mock-treated and AtSC-treated plants compared to those treated with LjSC (Fig 1C). These results show that Col-0 can be protected against the detrimental activity of R401 by bacterial communities, and this protection is SynCom-specific.

Protective *At*-SynCom function is suppressed by R401 via exometabolite-mediated inhibition of SynCom members

The decreased bacterial load of R401 on Col-0 roots in the presence of other bacteria may be a consequence of microbial antagonism. To test whether individual strains display antagonistic activity towards R401, we performed an *in vitro* pairwise antagonistic assay in which a producer strain that is dripped on top of a solidified medium produces exometabolites that diffuse into the medium and inhibit the in-agar growth of a target strain, resulting in a clear and quantifiable halo of inhibition ([33]; Materials and Methods). We found no profound effect of AtSC6 and LjSC6 strains on in-agar growth of R401, with only one *Bacillaceae* strain (AtRoot131) displaying mild inhibitory activity towards R401 (S2 Fig). However, in the reverse experiment in which R401 was the producer strain, most commensal bacteria (15/17 AtSC6

strains; 15/17 LjSC6 strains) were severely inhibited based on diameter measurement of the inhibition zone formed around the R401 colony (Fig 1D). This result corroborates a recent report identifying R401 as the most antagonistic bacterial isolate of the *At*-RSPHERE culture collection [33]. Notably, the same study revealed that >70% of R401 inhibitory activity *in vitro* was explained by the production of two molecules, namely the antimicrobial 2,4-diacetylphloroglucinol (DAPG) and the iron-chelating molecule pyoverdine that restrict the growth of other bacteria via direct antimicrobial activity and competition for available iron, respectively [33]. When we repeated our inhibition assays using the corresponding R401 $\Delta pvdY\Delta phlD$ double mutant impaired in both pyoverdine and DAPG production (R401mut), we confirmed the significant decrease of antagonism towards *At*-derived and *Lj*-derived commensal bacteria used in our study (Fig 1D). Strikingly, although R401mut retained its detrimental activity on Col-0 plants when mono-inoculated onto the seedlings (Fig 1A, B), this detrimental phenotype was now fully rescued by AtSC5 and AtSC6, indicating that *At* SynCom inability to protect against wild-type R401 (R401wt) reported above (Fig 1A) was causally linked to R401 exometabolite-mediated inhibition of AtSC5 and AtSC6 members (Fig 1A, B). These results show that protection by *At* SynComs depends on the absence of pyoverdine and DAPG production by R401, whereas protection by *Lj* SynComs occurs irrespective of their presence or absence. Based on these findings we conclude that there are SynCom-specific differences in the ability to restrict R401 root colonization and detrimental activity.

R401-induced bacterial community shift is SynCom-dependent and requires DAPG and pyoverdine production

To assess R401-induced remodeling of SynCom composition *in planta*, we profiled bacterial community composition in Col-0 roots colonized by AtSC6 or LjSC6 one week after infection with either R401wt, R401mut, or a mock solution. 16S rRNA gene amplicon sequencing and subsequent beta-diversity analysis (Bray-Curtis dissimilarities) revealed that R401wt-treated root samples differed from mock-treated and R401mut-treated samples, which cluster together (Fig 2A). The R401wt-induced shift in SynCom composition was clearer for the AtSC6

community compared to LjSC6 (S3A and B Fig; 32.8% with $p=0.001$ and 12.4% with $p=0.12$ of variance explained by R401-treatment, respectively, PERMANOVA), suggesting that LjSC6 is less affected by R401wt invasion than AtSC6. This differential impact of R401 on the communities observed *in planta* could be largely recapitulated in an *in vitro* experiment with liquid cultures in which R401 was added either at culture start or to a stabilized SynCom (S4 Fig), revealing that changes in composition were AtSC6-specific (S5A and B Fig). This is consistent with the idea that R401-induced bacterial community remodelling is largely host-independent and is therefore primarily explained by direct microbe-microbe competition.

We next calculated the relative abundance (RA) of individual strains within root-associated AtSC6 and LjSC6 and determined those strongly affected by R401. In AtSC6, strains AtRoot83 (*Alcaligenaceae*, log₂ FC 2.19), AtRoot935 (*Flavobacteriaceae*, log₂ FC 0.45), AtRoot101 (*Intrasporangiaceae*, log₂ FC -0.49), AtRoot68 (*Pseudomonadaceae*, log₂ FC -0.38), and AtRoot142 (*Rhizobiaceae*, log₂ FC 2.85) with RA >1% were significantly affected by R401wt compared to mock treatment ($p<0.05$) (Fig 2B), and this effect was abolished when R401mut was used. In contrast, none of the LjSC6 strains were significantly affected by either R401wt or R401mut compared to mock-control samples. Inspection of R401 levels based on RA in root samples revealed that R401wt efficiently invaded roots pre-colonized by AtSC6, and this colonization phenotype was partially impaired for R401mut (Fig 2C). This indicates that R401 requires production of DAPG and pyoverdine for invading AtSC6 and dominating in roots, consistent with results obtained with a soil-based gnotobiotic system and a different *At*-derived SynCom [33]. However, in contrast to these *in planta* observations, R401wt failed to become a stable member when added to already established liquid *in vitro* AtSC6 communities (S6A Fig), which may suggest that the host environment constitutes a condition that provides a competitive advantage for R401, which, however, can be challenged by more competitive commensals. In contrast, both R401wt and R401mut failed to invade LjSC6 pre-colonized roots (Fig 2C).

Taken together, these data indicate that R401-induced bacterial community shift (enabled through production of DAPG and pyoverdine) is required for R401 invasion, and that LjSC6

fully prevents R401 establishment on the roots likely due to the presence of specific strains competing with R401, thereby blocking its root invasion and preventing disease.

Swapping the *At*-SynCom *Pseudomonas* commensal with the *Lj*-SynCom strain confers protection against R401wt by AtSC6

We next aimed to narrow down which commensals of AtSC6 and LjSC6 drive R401 root colonization phenotypes and disease outcome. In the AtSC6 context, the RA of the *At* commensal *Pseudomonas* strain AtRoot68 was decreased in the presence of R401wt (Fig 2B), and the opportunist was abundant and caused plant disease symptoms (Fig 1A and B, 2C). However, AtRoot68 remained unaffected within AtSC6 when R401mut was used for infection (Fig 2B). In the LjSC6 context, the RA of the *Lj* commensal *Pseudomonas* strain LjRoot59 was unaffected (*in planta*) or was strongly increased (*in vitro*), while R401wt levels stayed low and plants healthy (Fig 1A and B, 2B, S6B Fig). Based on these observations, we suspected that competition between individual strains within the *Pseudomonas* genus may determine the colonization success of R401 within a root-associated community. To test this hypothesis, we swapped the two commensal strains in the SynComs and found that LjRoot59 was indeed necessary and sufficient to drive SynCom protective function against R401wt *in planta* (Fig 3A and B). Normal plant growth was detected after R401wt treatment when LjRoot59 was present in AtSC6 instead of AtRoot68, and plants wilted when AtRoot68 was present in LjSC6 instead of LjRoot59 or when no other strain was added (Fig 3A and B). Amplicon sequencing of root samples and quantification of bacterial RA further corroborated that the presence of LjRoot59 prevents R401wt from establishing as part of the community (S7 Fig). Given that plant performance was significantly better in the presence of LjSC6 even without LjRoot59 compared to axenic plants (Fig 3B), it is possible that other commensal strains within LjSC6 also contribute to protective activity towards R401 *in planta*. These results show that in taxonomically diverse root-associated communities, the colonization success of an opportunistic pathogen depends on the presence of another individual belonging to the same genus.

***Pseudomonas*-mediated protection against R401 is strain-specific**

Next, we tested 15 *Pseudomonas* members of our two culture collections (S2 Table) to investigate how general or specific the competition between the strains was. *In vitro* halo of inhibition assays showed that the growth of five strains, irrespective of their host origin, was inhibited by R401wt (including AtRoot68 but not LjRoot59), but not or partially by R401mut (S8 Fig). Conversely, five other isolates inhibited R401wt growth *in vitro* (not AtRoot68 or LjRoot59), and those five plus four more also inhibited R401mut (Fig 4A). Interestingly, when plants were co-cultivated with these 15 commensal *Pseudomonas* strains separately, ten of them exhibited protection against R401 (Fig 4B). This protective ability did not correlate with the host of origin of the strains, nor with their antagonism towards R401 *in vitro* (Fig 4C). Furthermore, there was no significant correlation between the phylogeny of the strains, which was based on the full-length 16S rRNA gene sequences, and the protection *in planta* (Pearson's product-moment correlation 0.45, $p=0.092$; S3 Table). Of note, two of the commensals phylogenetically close to R401, LjRoot54 and LjRoot154, were slightly detrimental on Col-0 based on the shoot appearance, however, not to the same extent as R401, and the disease phenotype did not become stronger upon R401 infection, indicating that they were still able to outcompete R401.

***Pseudomonas*-mediated protection against R401 is predicted to occur via resource competition**

The variation in the observed competitiveness of the *Pseudomonas* strains *in planta* could be a result of strain-specific variation of growth advantage and proliferation. Given that protective ability did not correlate with the strains' sensitivity or resistance to R401, we hypothesized that resource competition might explain *Pseudomonas*-mediated protection against R401. We first measured the growth of these strains in a medium supplemented with carbon sources abundantly secreted into the rhizosphere by plants (artificial root exudates: glucose, fructose, sucrose, citric acid, succinate, lactate, glutamate, alanine, and serine; [17, 35]). From the

growth curves (S9A and B Fig), we derived the maximum growth rates and found that they vary among the *Pseudomonas* isolates and do not correlate with protective activity against R401 *in planta* (Wilcoxon rank sum test, $p=0.76$) (S9C Fig, S4 Table).

Since the *Pseudomonas* strains can generally use these synthetic exudates, their differential competitiveness on roots may be due to their capacity to use other compounds. Therefore, we used rhizoSMASH (<https://git.wur.nl/rhizosmash>) to predict the bacteria's catabolic potential based on their whole-genome sequences. In total, we found catabolic gene clusters (CGCs) for the catabolism of 34 distinct compounds (Fig 5, S5 Table). The number of these CGCs varied between 0 and 4 per compound across the *Pseudomonas* genomes (Fig 5). We measured how similar or dissimilar the CGC profiles of the strains were compared to that of R401 (S10 Fig) and tested if a higher similarity correlated with protective activity *in planta*. We found a moderate correlation (Pearson's product-moment correlation 0.563, $p=0.029$) between the protective activity and the similarity of the genomic potential for catabolic activity between the protective strains and R401 (S6 Table). In addition, the distances of CGC profiles of protective strains to R401 were significantly smaller than the distances of non-protective strains (Wilcoxon rank sum test, $p=0.027$).

These results may suggest that bacteria with a similar catabolic potential as R401 may possess a competitive advantage in co-cultivation *in planta*, thereby representing one possible mechanism by which *Pseudomonas* commensals might restrict R401 colonization on the roots.

DISCUSSION

Within the concept of the holobiont [36], the microbiota is considered a genetic and thus functional extension of the host gene repertoire. Therefore, it is reasonable to assume that plants benefit from accommodating a tailored subpopulation of micro-organisms. The microbial species may in turn experience an advantage from colonizing a plant species that provides a specific structural and chemical environment. In our study, we investigated if taxonomically diverse SynComs of different host origin were able to provide protection against the detrimental activity of the opportunistic *Pseudomonas* strain R401 on roots of *At Col-0*.

We found that within a controlled agar-based growth system, the presence of *Lj*-derived SynComs prevented the plants from wilting after infection with R401, whereas taxonomically equivalent *At*-derived SynComs failed to do this efficiently (Fig 1A and B). The disease phenotype correlated with a high detectable titer of R401 on the roots (Fig 1C and 2C), which could be a result of antagonistic activity of the strain towards the commensals, rendering it more competitive and dominant. R401 was originally isolated from healthy-looking *Col-0* plants as part of the *At* culture collection [30]. Its dominance is possibly more pronounced within the *At* SynCom since it may have developed a competitive advantage among other *At*-associated bacteria. Consistent with that, R401wt-induced remodelling of AtSC6 composition was directly linked to both invasion and disease and requires production of pyoverdine and DPAG (Fig 1 and 2). Additionally, in a previous study and a similar experimental setup, R401wt root colonization was also only slightly restricted by commensal *At* SynComs [31], although these SynComs consisted of only four members and did not contain *Pseudomonas* strains. However, since R401wt inhibited *in vitro* growth of both *At*- and *Lj*-derived diverse bacterial taxa (Fig 1D), *in vitro* antagonism is not sufficient to predict dominance *in planta*.

Relative quantification of the bacteria within root-associated SynComs revealed the impact of R401 on the other community members (Fig 2B). A significant effect of R401 on a diverse 18-member SynCom has also been reported for a soil-based system [33]. In those experiments,

however, no other *Pseudomonas* strain was included, and R401 did not cause wilting symptoms despite high relative abundance. Interestingly, in our system, the abundance of the commensal *At Pseudomonas* strain AtRoot68 was strongly decreased in AtSC6, and the *Lj Pseudomonas* counterpart LjRoot59 stayed unaffected, while R401wt failed to colonize within LjSC6 (Fig 2C). Since here the *Pseudomonadaceae* members make up at least 30% of the abundance within the communities, we regarded this differential impact of R401wt as important. We suspected that intra-genus dynamics could play a decisive role for R401wt colonization success. Indeed, when testing the protective function of several individual *At* and *Lj Pseudomonas* isolates *in planta* (Fig 4), we found that other *Pseudomonas* commensals were also capable of suppressing the detrimental effect of R401wt on *Arabidopsis*. However, this trait was independent of the strains' origins, phylogeny, and *in vitro* antagonism towards R401wt (Fig 4C). These results suggested that, besides the activity of secreted antimicrobial compounds, intra-genus competition may determine colonization success.

Besides direct antagonisms, competition for resources contributes to microbial population dynamics [37] when the amount of nutrients is limited, or when only certain types of nutrients are available. Organisms with a corresponding resource-usage profile then have a competitive advantage over those with more inapt profiles, and the latter could benefit from cross-feeding on metabolic products secreted by other species. In line with this, it was demonstrated for phyllosphere-associated microbiota members that bacteria-bacteria interaction outcomes *in planta* can be predicted by the carbon source utilization profiles combined with genome-based metabolic modeling of the strains and the overlap of those [38]. This study found mostly negative interactions between strains *in vitro*, which corresponded to niche overlap. An increase of positive interactions *in planta* (and other aspects not covered by the model) was explained by cross-feeding, additional metabolic preferences, signaling, host immune response, and fluctuating environmental conditions. Moreover, Pacheco-Moreno et al. recently inferred from the different gene sets of *Pseudomonas* strains isolated from the rhizosphere of two different barley cultivars distinct bacterial preferences for primary plant metabolites, and

linked those to the abundance of certain metabolites in the root exudates of the plant genotypes [39]. When we inspected the genomes of our *Pseudomonas* isolates, we found that strains with protective activity against R401 *in planta* possess similar catabolic gene clusters as R401 itself (Fig 5, S5 and S6 Tables), which may be a first prediction for a possible niche overlap also in our microbiota reconstitution system. In future experiments, the presence of the corresponding CGC-related compounds in root exudates and their role in competitive root colonization should be explored.

Host involvement in interactions between plants and diverse *Pseudomonas* strains has also been described for a phyllosphere community [22]. In our study, R401wt and commensal dynamics are affected by the *At* host as well, since R401wt invasion of the root-associated AtSC6 was possible, whereas invasion within an established liquid community failed (S6 Fig). The relevant plant factors remain to be shown, and likely comprise root-exuded compounds, including nutrients and signalling molecules that affect both the commensals and the detrimental strain, and parts of the plant immune system.

Our findings corroborate results from other studies on protective activities of commensal strains against pathogenic or opportunistic bacteria in roots, and this is often linked to microbe-microbe dynamics. For example, several studies with tomato and the bacterial wilt-causing strain *Ralstonia solanacearum* demonstrated that microbial competition can lead to the successful exclusion of this pathogen, e.g., correlating with the number [40] and diversity [41] of co-inoculated rhizobacterial strains and the number of utilized carbon sources. Using resource competition networks for SynComs of non-pathogenic *Ralstonia* spp., Wei et al. showed that, when all species can use resources similarly, and the resource niche overlaps with the pathogen's, pathogen titer and thus disease decrease [42]. Similarly, in the human gut, commensal bacterial strains only protected against pathogenic invaders if they, in combination with others, exhibit a community nutrient utilization profile that is competitive with the pathogen's [43]. However, if a single strain exhibits such a profile, it may be sufficient for

this nutrient blocking. Importantly, our strain-swapping and -dropout experiments showed that LjRoot59 is sufficient to protect Col-0 from being colonized by R401wt (Fig 3). Of note, we cannot exclude that a combination of mechanisms, including resource competition, induction of host immune response, or *in planta* expression of antimicrobials may cause the restriction of R401wt root colonization. Nevertheless, our data indicates that even within a diverse community of different bacterial taxa, one specific strain with relevant properties can outcompete a detrimental one.

In addition to R401, other plant opportunistic *Pseudomonas* strains have previously been isolated from healthy plants and other environments [44, 45], supporting the idea that the microbiota members exert both antagonistic and competitive activities to keep the pathogen titers low in natural environments. For the detrimental strain N2C3 it was shown that, among diverse *Pseudomonas* isolates, only highly related *Pseudomonas* strains protect *At*, and that they require a functional response regulator ColR that allows for root colonization [46]. In contrast to R401 (Fig. 1C and 2C), N2C3 keeps its colonization ability even in the presence of a protective strain [46], indicating different modes of competition of these two detrimental strains, although the different experimental setups may play a role as well. Interestingly, an avirulent mutant of the pathogen, in which the genes for the phytotoxic agent were deleted, was not able to protect plants against the wild-type strain, suggesting the requirement of additional genetic components, despite the already high phylogenetic similarity [46]. Furthermore, besides the intra-genus competition reported by us and by others, individual distantly related bacterial microbiota isolates have been shown to protect plants against pathogens, albeit through mechanisms other than direct resource competition [47]. In line with this, other taxa than *Pseudomonadaceae* may contribute to protective activity in our system as well, since LjSC6 leads to slightly better plant performance than axenically grown plants, even without the *Pseudomonas* commensal LjRoot59 (Fig 3B).

In summary, our experiments show that an individual beneficial strain can be sufficient to outcompete an opportunistic relative, either alone or within a taxonomically diverse microbial community. This is important because it may indicate that, in field settings under certain circumstances, i) the beneficial species can persist, and ii) the surrounding members can stabilize the community as a whole. Understanding such dynamics is crucial for engineering stable and beneficial microbial consortia [48]. The difference in relative abundance of certain commensals and opportunistic strains in a competitive situation is likely due to selective carbon source preferences and niche overlap. This concept provides important implications for sustainable agricultural processes. In further studies, especially in greenhouse or field conditions, testing the function of additional host-specific micro-organisms in protection of their host against other, non-adapted but detrimental microbial species will be necessary to determine generality of our findings.

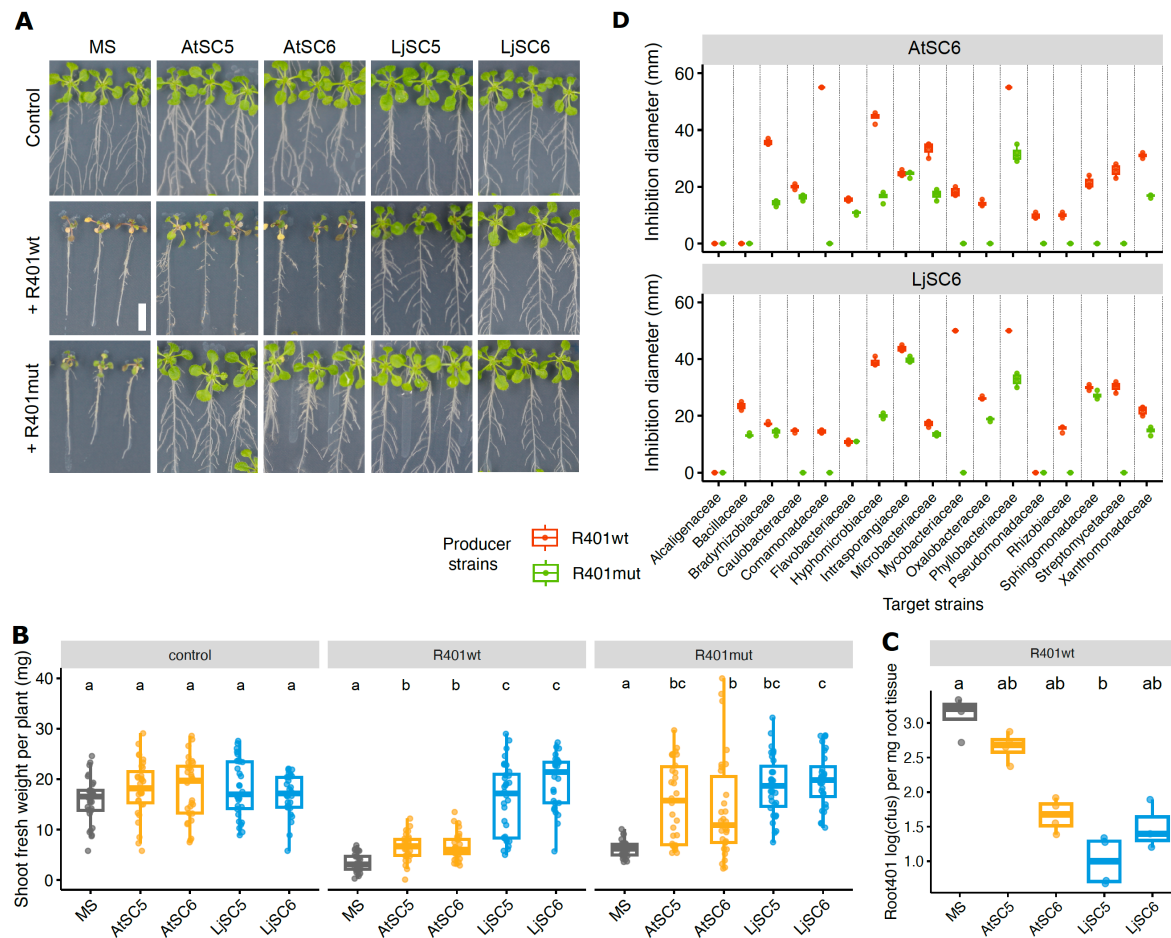


Fig 1: SynCom-specific detrimental effect of R401wt and R401mut on Col-0 plants.

(A) *Arabidopsis thaliana* Col-0 phenotypes grown on MS agar plates alone (MS) or in co-cultivation with the indicated SynComs (AtSC5, AtSC6, LjSC5, LjSC6), and after treatment with mock (Control), R401 wild-type (R401wt) or R401 $\Delta pvdY \Delta phlD$ (R401mut) strain. Scale bar corresponds to 1 cm. **(B)** Shoot fresh weight of plants shown in A. **(C)** Number of R401wt colony forming units (cfus) at 2 days post infection on Col-0 roots co-cultivated with indicated SynComs. **(B and C)** identical letters indicate no significant statistical differences (Kruskal-Wallis followed by Wilcoxon rank sum test and Bonferroni adjustment; p-value <0.05). **(D)** Inhibition diameter formed around either a R401wt or a R401mut colony (producers) on a lawn of the indicated individual commensal strains (targets) on an agar plate.

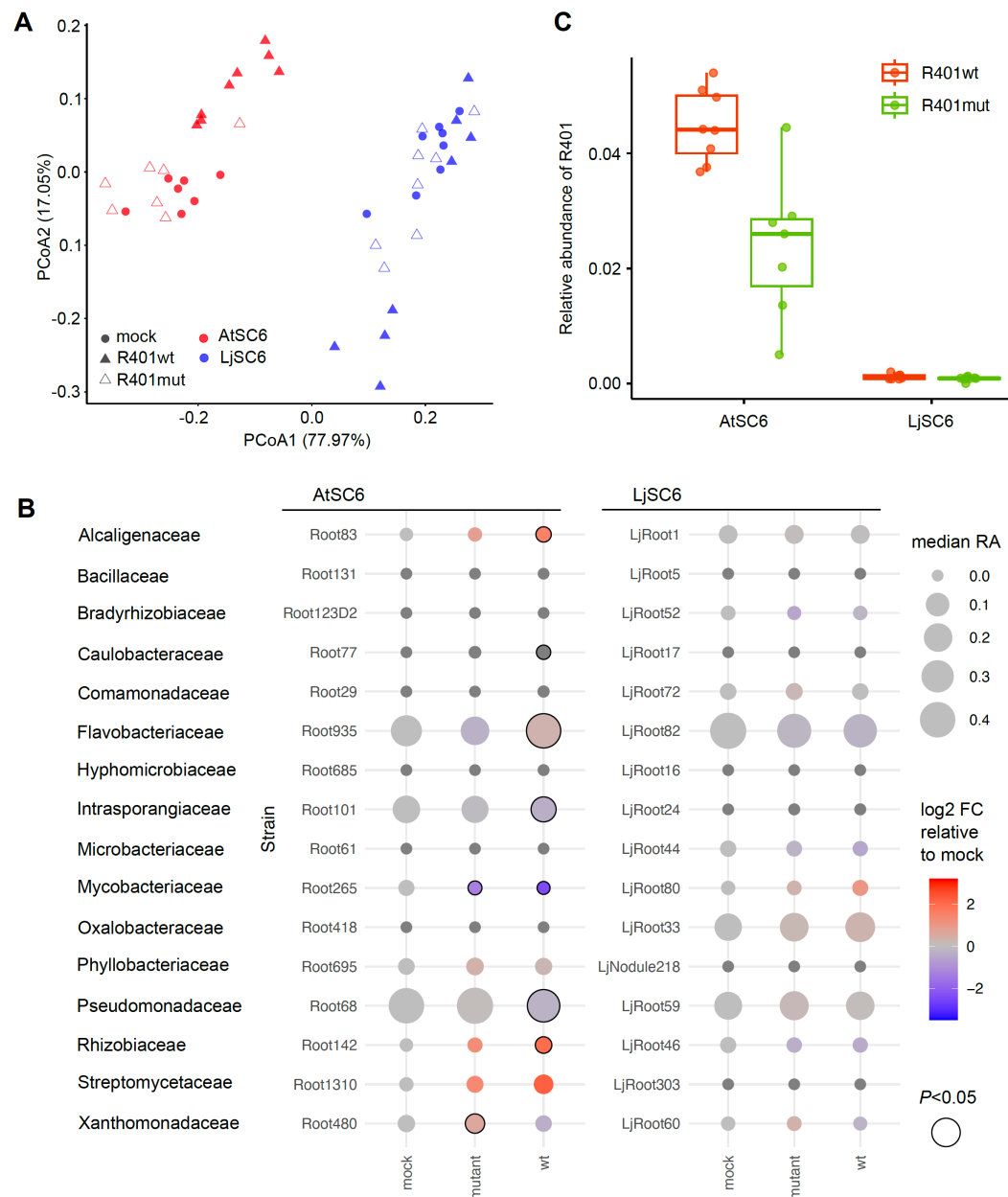


Fig 2: R401-induced community shifts in AtSC6 and LjSC6 on Col-0 roots. (A, B and C) Bacterial community diversity and composition on roots of 21-day old Col-0 seedlings after initial co-cultivation with AtSC6 or LjSC6 and subsequent treatment with either mock, R401wt, or the R401 $\Delta pvdY$ $\Delta phlD$ mutant at 14 days. **(A)** Principal Coordinate Analysis (PCoA) of Bray-Curtis dissimilarities of bacterial 16S rRNA amplicon sequences of root samples. **(B)** Quantification of individual bacterial strains within SynCom AtSC6 or LjSC6. Circle size corresponds to median relative abundance (RA). Color gradient indicates log2-transformed fold change of abundance relative to mock condition. Black circle outline indicates significant fold change (Wilcoxon rank sum test, $p < 0.05$). Strain IDs and bacterial families are indicated. **(C)** Relative abundance of R401wt and R401mut within AtSC6 or LjSC6 on Col-0 roots.

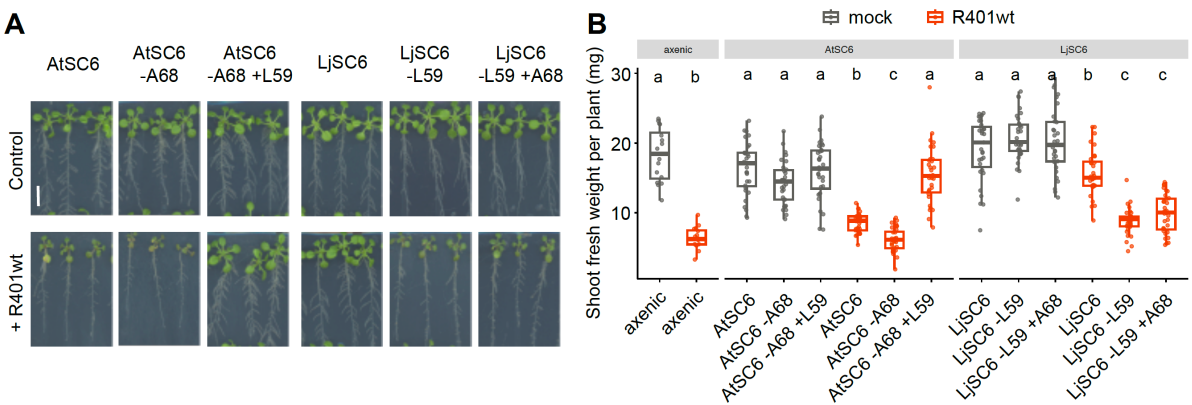


Fig 3: Rescue of AtSC6 protective activity after *Pseudomonas* commensal swap. (A) Col-0 phenotypes grown on agar plates in co-cultivation with the indicated SynComs after treatment with mock (Control) or R401 wild-type (R401wt). AtSC6-A68, AtSC6 without AtRoot68; AtSC6-A68+L59, AtSC6 without AtRoot68 but with LjRoot59; LjSC6-L59, LjSC6 without LjRoot59; LjSC6-L59+A68, LjSC6 without LjRoot59 but with AtRoot68. Scale bar corresponds to 1 cm. **(B)** Shoot fresh weight of plants shown in C. Identical letters indicate no significant statistical differences (Kruskal-Wallis followed by Wilcoxon rank sum test and Bonferroni adjustment; p-value <0.05).

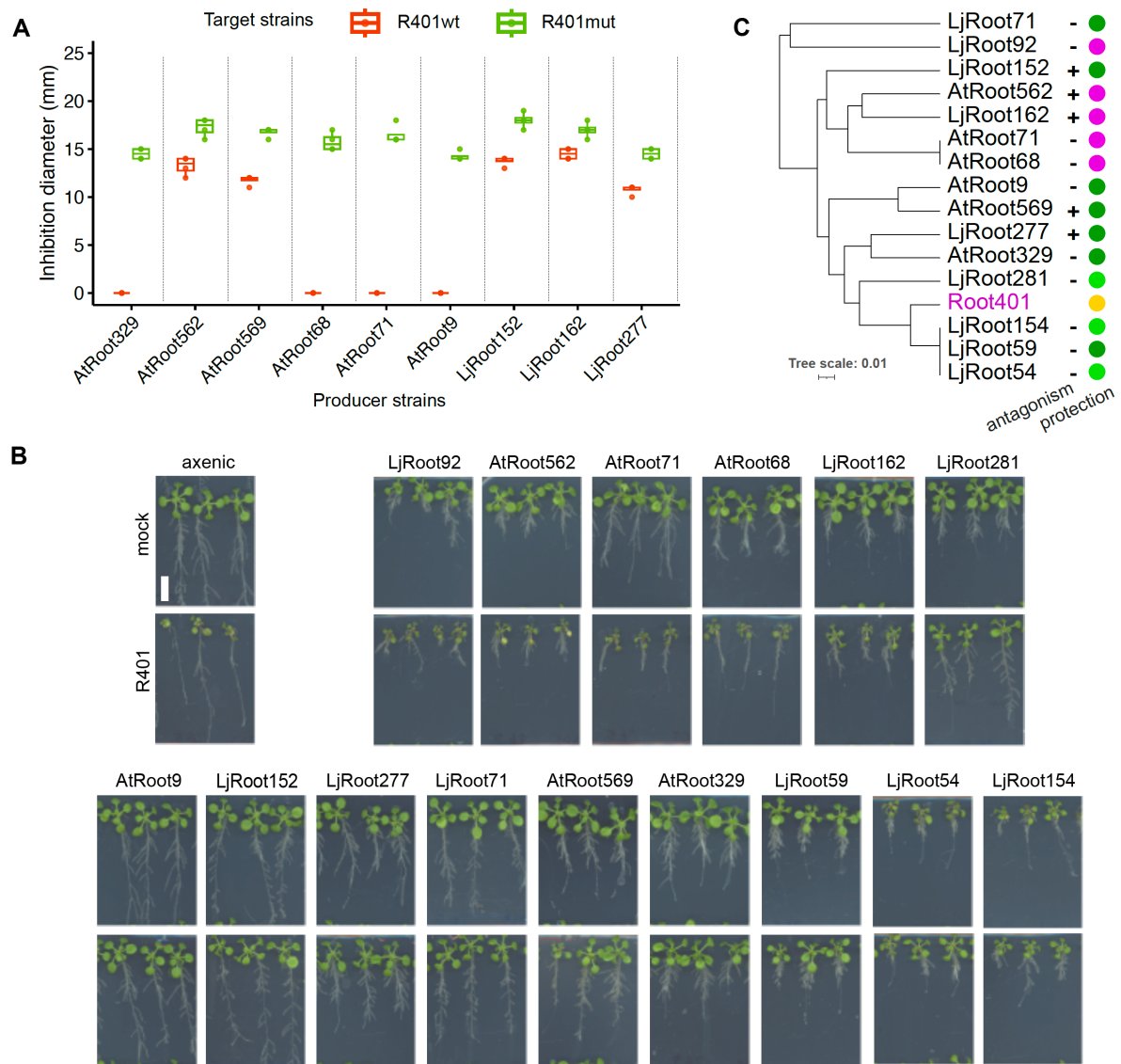


Fig 4: Competition between R401 and individual *Pseudomonas* strains *in vitro* and *in planta*. (A) Inhibition diameter formed around colonies of the indicated nine individual *Pseudomonas* commensal strains (producers) on agar plates on a lawn of either R401wt or R401mut (targets). The other six *Pseudomonas* commensals did not inhibit R401 growth. (B) Col-0 phenotypes in co-cultivation with the indicated individual *Pseudomonas* strains after treatment with mock (upper panels) or R401wt (lower panels). Scale bar corresponds to 1 cm. (C) Phylogenetic tree of *Pseudomonas* strains based on full-length 16S rRNA gene sequence. Presence (+) or absence (-) of antagonistic activity by the commensal strains towards R401wt in inhibition assays is indicated. Filled circles indicate protection (green) and no protection (magenta) of Col-0 by these strains against R401wt when in binary *in planta* assays (panel B). Light green color indicates either medium protection or slightly worse plant shoot appearance under mock conditions.

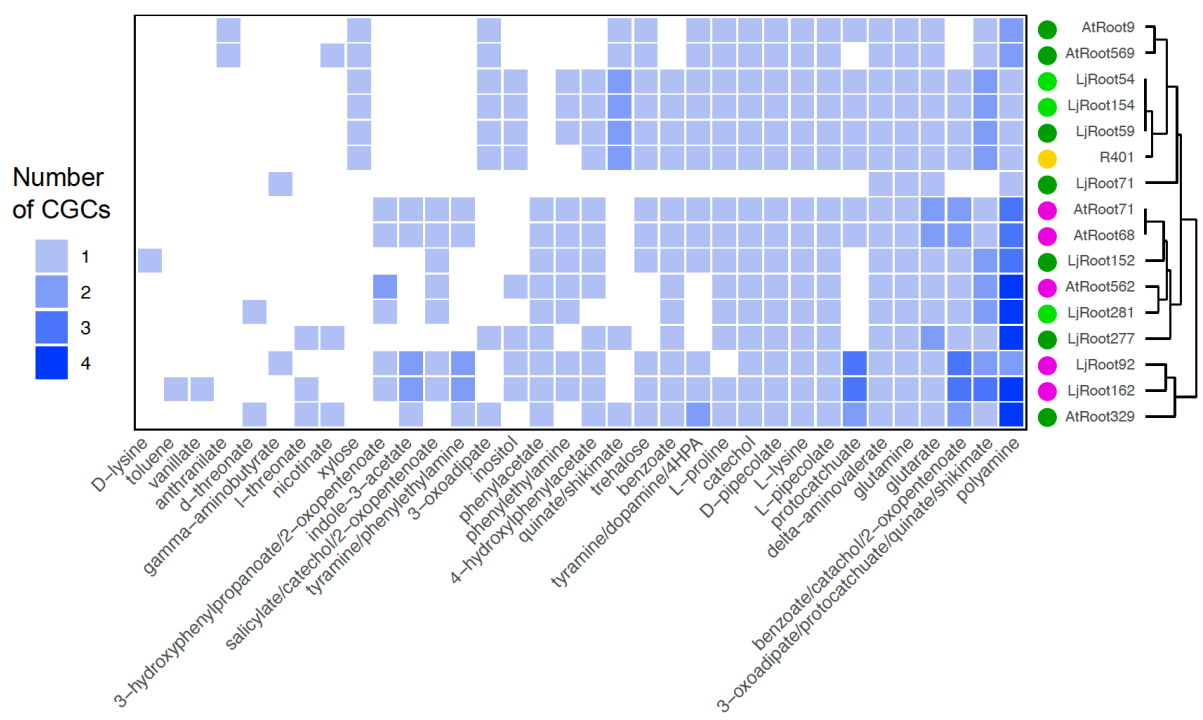


Fig 5: Bacterial catabolic potential. Number of catabolic gene clusters (CGCs) related to the catabolism of the indicated compounds in individual *Pseudomonas* strains, based on a survey of their whole-genome sequences.

MATERIALS AND METHODS

Bacteria

All commensal bacteria were obtained from the *At*-RSPHERE and *Lj*-SPHERE culture collections [17, 30]. The compositions of the SynComs are listed in the [S1 Table](#). In brief, 17 strains were chosen per SynCom, representing the 17 bacterial families shared between the two culture collections. The tested *Pseudomonas* strains are listed in the [S2 Table](#). Transgenic Root401 and Root401 *pvdY phlD* mutant both carrying a chromosomal gentamycin resistance, were kindly provided by Felix Getzke [33]. Bacteria were grown at 25°C in 15g/L tryptic soy broth (TSB) or TY (5g/L tryptone, 3g/L yeast extract, 10 mM CaCl₂), with 15g/L Bacto Agar (Difco) for solid media. Gentamycin was added where required to a final concentration of 50 ug/mL.

Co-cultivation of plants and bacteria on agar plates

Arabidopsis thaliana Col-0 wild type seeds were surface-sterilized by incubation in 70% ethanol for five minutes, followed by two steps of one-minute incubation in 100% ethanol and five steps of washing in sterile MilliQ water. Seeds were kept in approx. 200 uL of water to imbibe and in the cold and dark for stratification for four days. Bacterial liquid cultures were prepared by inoculating 3 mL of 50% TSB medium or TY medium with a colony of bacteria recently streaked onto agar plates. After two days of growth, cells were harvested by centrifugation, washed twice in 10 mM magnesium sulfate (MgSO₄), and resuspended in 3 mL of MgSO₄. OD600 was measured, and SynComs were prepared such that each strain in the mix had a final OD600 of 1. Aliquots of 300 uL were taken of each SynCom, and of 50 uL of each individual strain, frozen and kept at -80°C until further processing for sequencing. To prepare agar plates, SynComs were added to autoclaved, hand-warm 0.5x MS medium (2.22 g/L MS including vitamins (Duchefa), 0.5 g/L MES, pH adjusted to 5.7 with KOH) containing 10 g/L Bacto agar (Difco Agar, Becton Dickinson) to a final OD600 of 0.005. Per 12cm x 12cm squared petri dish, 50 mL of medium only or medium-SynCom mix were used. 20 sterilized seeds per plate were placed onto the solidified medium in two rows, plates sealed with M3

Micropore tape and placed vertically in a light cabinet with a 10/14h light-dark cycle. After two weeks, plates were opened inside a clean bench, 15 mL of mock (10 mM MgSO₄) or R401 suspension (OD 0.0001 in 10 mM MgSO₄) were carefully added per plate and plates tilted to distribute the liquid. After 10 min of incubation, the liquid was removed and seedlings transferred to new, sterile 0.5x MS agar plates. Samples for viable counts of R401 were taken at 2 dpi (days post infection), and samples for amplicon sequencing at 7 dpi. Pictures of plants were taken around 7 and around 14 dpi. See [S1 Figure](#) for schematic workflow.

Viable colony counts of R401

Plant shoots were cut off the roots with a scalpel on the agar. Roots of 2-4 plants were transferred to one well of a 12-well plate with sterile 10 mM MgSO₄ and washed for a few seconds by swirling them with forceps. Roots were dried by padding on a piece of sterile Whatman paper, transferred to a prepared sterile 2-mL screw-cap tube containing grinding beads, weighed, and put aside on a rack. Four replicates per plate were harvested, and this was repeated for all plates. Roots were homogenized by subjecting the tubes to 2x 6200 rpm with a 15 second break in the Precellys Homogenizer. 400 µL of 10 mM MgSO₄ was added to each tube, and the homogenization procedure repeated. In a 96-well PCR plate, 100 µL of each root extract were transferred to one well, and serial dilutions of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ were prepared in 10 mM MgSO₄. 10 µL per condition and dilution were spotted onto a 50% TSB squared plate containing 50 µg/mL gentamicin, and the plate tilted such that the liquid was distributed across half the width of the plate. Two technical replicates were prepared, and colony forming units (cfus) of transgenic, gentamicin resistant R401 counted after 2-3 days. Only two of the SynCom strains, *Flavobacterium* AtRoot935 and LjRoot82, are somewhat resistant to gentamycin, meaning that they grow on the selection plates. However, the bright orange color of those colonies could be distinguished from the white R401 colonies.

Sampling and amplicon sequencing

Plant shoots were cut with a sterile scalpel and forceps and weighed one by one. Roots of 3-4 plants were removed with sterile forceps and washed briefly by swirling in one well of a 12-well plate containing sterile 10 mM MgSO₄. Roots were padded on sterile Whatman paper to dry and transferred into Lysing Matrix E tubes (FastDNA spin kit for soil, MPBiomedicals), and frozen in liquid nitrogen immediately. With four samples per plate, and two replicate plates per condition, there were eight replicates per condition in total. Samples were stored at -80°C. DNA extraction, library preparation, and amplicon sequencing of the 16S rRNA v5-v7 gene region with the in-house Illumina MiSeq platform at the MPIPZ Department of Plant-Microbe Interactions was performed as described previously [17].

Analysis of sequencing data

Amplicon sequencing data analysis was performed as described previously [17]. In silico depletion of R401 was achieved by removing sequencing reads for R401 from the count table prior to computation of relative abundances. This approach was applied to amplicon sequencing data from samples with plants treated with R401 or a mock solution for the Principal Coordinate Analysis of Bray-Curtis dissimilarities and for the abundance plot and fold change calculation.

***In vitro* inhibition assays**

The potential of a producer strain to inhibit the growth of a target strain through the production of diffusible antimicrobial compounds was tested. This was done by spotting the producer strain on top of solidified agar in which the target strain was growing and assessing the formation of a halo (lack of growth of the target strain) around the producer strain. Cultures of individual bacterial strains were grown for two days in TY medium. Strains were harvested by centrifugation, washed twice in 10 mM MgSO₄, and adjusted to OD₆₀₀ of 0.5 in TY medium. To prepare agar plates with the target strains, 50 µL of bacterial culture were added to 50 mL of autoclaved, hand-warm TY agar to obtain OD₆₀₀ of 0.0005, and the mix was distributed into four round petri dishes (6-cm diameter). After solidification, 10 µL of the producer strain

with OD600 of 0.1 were dropped in the middle of the plate and left to dry. Plates were incubated at 25°C for 2-5 days, and halo formation was recorded by taking pictures of the plates with a scanner. Halo size (“inhibition diameter”) was measured as the diameter of the halo crossing the spotted producer strain. Fiji software was used when possible, and manual measurement with a ruler was applied when growth of the target strain was weak and contrast adjustments in the software were not sufficient to visualize the halo on a digital image.

***In vitro* co-cultivation of SynComs with R401**

To assess the effect of R401 on the composition of bacterial SynComs after growth in culture, SynComs were incubated with or without R401 in liquid medium for multiple days. See [S4 Figure](#) for schematic workflow. Bacterial strains were grown in liquid TY medium for 3 days, then diluted 1:10 in fresh medium and grown for another day. Strains were harvested by centrifugation, washed in 5 mL 10 mM MgSO₄, and resuspended in 2-5 mL MgSO₄, based on visual assessment of culture density. All 17 strains of a specific SynCom were combined in a 50-mL Falcon tube, using 1 mL per strain. OD600 was measured, SynComs diluted to OD600 of 0.1 in a 50-mL tube and split into two cultures. To one tube, R401 was added to a final OD600 of 0.006, which corresponded roughly to the OD600 of each individual strain in the mixture. Aliquots of 300 uL were taken of all conditions (AtSC6, AtSC6+R401, LjSC6, LjSC6+R401) for timepoint t0. Each culture was divided into 4x 5-mL cultures in culture tubes and incubated shaking at 25°C. After three days, samples were taken from all cultures and frozen at -80°C. Then, OD600 was measured of all cultures and cultures diluted in new tubes with TY to a final OD600 of 0.1. For the cultures without R401, two batches were prepared, and the amount of freshly grown R401 culture added to one batch for a final OD600 of 0.006. Cultures were incubated shaking at 25°C for another three days. Aliquots of 300 uL of all tubes were taken and frozen at -80°C. DNA of all cultures was isolated using alkaline lysis. For this, samples were left to thaw at room temperature. Wells of a 96-well PCR-plate were filled with 20 µL buffer 1 (25 mM NaOH, 0.2 mM EDTA, pH 12), 12 µL of sample were added, the plate incubated at 95°C for 30 min, 20 µL of buffer 2 (40 mM Tris-HCl at pH 7.5) added to each well,

and the plate sealed and kept at -20°C until further processing (library preparation and amplicon sequencing, see above).

Phylogenetic tree

Multiple sequence alignment of the full-length 16S rRNA gene sequences of the *Pseudomonas* strains listed in the [S2 Table](#) was performed using the MUSCLE method in the CLC Workbench software. Hierarchical clustering was done with the UPGMA method, and phylogenetic tree visualization with the help of the Interactive Tree of Life webtool [34].

Bacterial growth curves

To measure growth curves, a minimal medium containing M9 salts (3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl), vitamins (0.4 mg/L 4-aminobenzoic acid, 1 mg/L nicotinic acid, 0.5 mg/L calcium-D-pantothenate, 1.5 mg/L pyridoxine HCl, 1 mg/L thiamine HCl, 0.1 mg/L biotin, 0.1 mg/L folic acid, 0.4 mg/L riboflavin, 0.2 mg/L cyanocobalamin, 0.05 mg/L lipoic acid), 1 mM MgSO₄, 0.1 mM CaCl₂, and carbon sources was used. The 5x stock of M9 salts was adjusted to pH 7.2 with NaOH before usage. The 100x vitamin mix was adjusted to pH 6.8 with KOH before usage. First, individual carbon sources were prepared such that the final concentration in the medium was 30 mM with respect to number of C atoms in the molecule. Stock solutions of the carbon compounds were prepared as 10x solutions. For the artificial root exudates, nine carbon sources (glucose, fructose, sucrose, citric acid, succinate, lactate, glutamate, alanine, and serine) were added in equal volumes to the medium, corresponding in total to 1/10 of the volume of the final medium. Bacterial cultures were grown over night in 50% TSB medium. Cultures were then washed once in minimal medium without carbon sources and OD600 adjusted to 0.5 in minimal medium. In a microtiter plate, 20 uL of each strain were added to a well containing 180 uL of minimal medium with carbon sources, yielding an OD600 of 0.05. Plates were incubated shaking at 25°C. Absorbance at 595 nm was measured with a plate reader (TECAN Infinite F50) at different intervals. The maximum growth rates for each

individual strain were calculated using the *fit_easylinear* function of the R *growthrates* package (<https://github.com/tpetzoldt/growthrates>).

Detection of catabolic gene clusters in bacterial genomes

Whole genome assemblies based on Illumina short read sequencing for the bacterial isolates are available from previous studies [17, 30]. We subjected these assemblies to the rhizoSMASH algorithm (available at <https://git.wur.nl/rhizosmash>, Yuze Li and Marnix Medema, Wageningen University and Research, The Netherlands) to detect catabolic gene clusters (CGCs) based on sequence similarity to publicly available bacterial genomes. CGC names with significant hits were extracted from the output data for each *Pseudomonas* strain. Euclidean distances were calculated based on the presence and number of the CGCs within the genomes relative to the R401 genome using the *dist* function of the R core *stats* package.

Data availability

Raw reads of amplicon sequencing data are available at the NCBI Sequence Read Archive under BioProject ID PRJNA1162794.

AUTHOR CONTRIBUTIONS

A.A. and K.W. performed experiments. A.A., K.W., and A.H.-B. analyzed the data. K.W. conceived the study. S.H. shared unpublished material. K.W. wrote the manuscript with input from all authors.

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SUPPORTING INFORMATION

- S1 Figure: Schematic showing experimental setup of agar-based co-cultivation assays.
- S2 Figure: Agar plate scans of halo assay with R401 as target.
- S3 Figure: CPCoA plots of root-associated AtSC6 and LjSC6 separately after R401wt and R401mut treatment.
- S4 Figure: Schematic showing experimental setup of SynCom cultivation in liquid culture and sampling times.
- S5 Figure: PCoA plots of R401-induced community shifts of AtSC6 and LjSC6 liquid cultures.
- S6 Figure: Bar plots showing relative abundance of *At* (A) and *Lj* (B) strains after cultivation as liquid cultures with and without R401.
- S7 Figure: Relative abundance of R401wt in root-associated SynComs with *Pseudomonas* drop-outs or swaps.
- S8 Figure: *In vitro* inhibition of *Pseudomonas* commensals by R401wt and R401mut.
- S9 Figure: Growth curves and rates of *Pseudomonas* strains in minimal medium with artificial root exudates.
- S10 Figure: Euclidean distance matrix of CGC numbers per strain.
- Supplementary tables:
 - S1 Table: SynCom compositions.
 - S2 Table: *Pseudomonas* isolates.
 - S3 Table: Distance indeces of *Pseudomonas* isolates to R401 16S gene.
 - S4 Table: Growth parameters of individual *Pseudomonas* strains.
 - S5 Table: Catabolic gene clusters detected in *Pseudomonas* genomes.
 - S6 Table: Euclidean distances of R401wt to the other *Pseudomonas* strains based on the presence of catabolic gene clusters.