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Supporting Information for

Co-functioning of bacterial exometabolites drives root microbiota establishment

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Material, data, and code availability

Detailed Material and Methods

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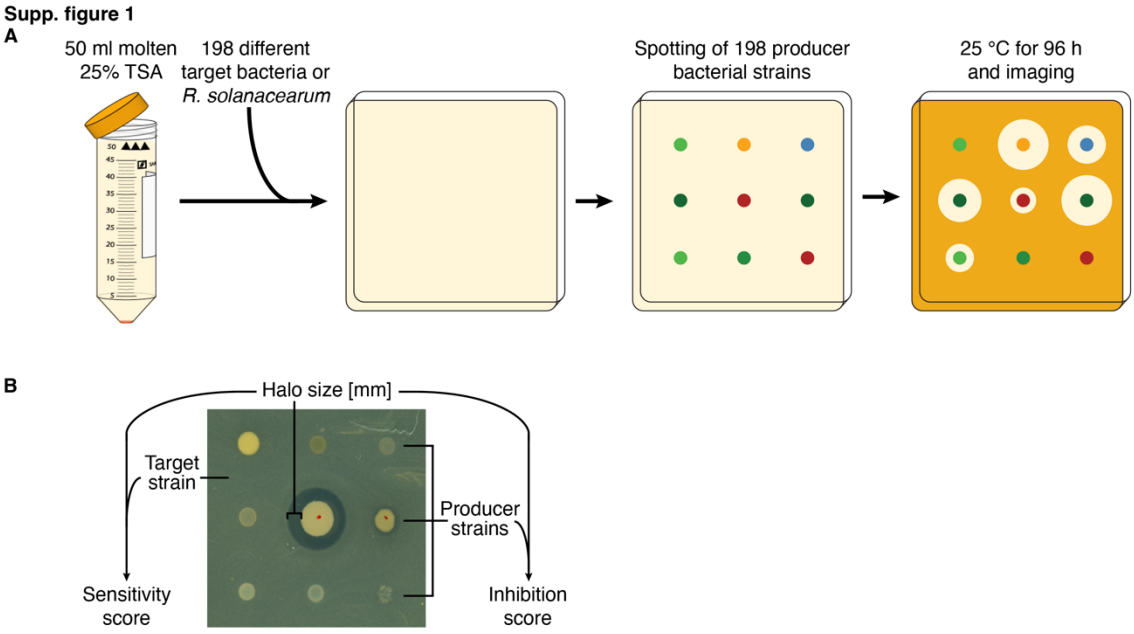


Figure S1 Flowchart and output example of the modified Burkholder assay (mBA) screen

(A) Schematic illustration of the screen for interbacterial interactions in a mBA. First, 100 µl of a bacterial solution from one strain are re-suspended in 50 ml of cooled, but molten 25% tryptic soy agar (25% TSA) and poured into a petri dish. Then, 24 bacterial strains are spotted on top of the solidified medium. The plates are incubated for 96 h at 25 °C. Pictures are taken and the size of the halo of inhibition is measured.

(B) An example agar plate depicting a halo of inhibition produced by a producer strain on the bacterial lawn of a target strain, grown in 25% TSA. Explanation of nomenclature used in Fig. 1.

Supp. figure 2

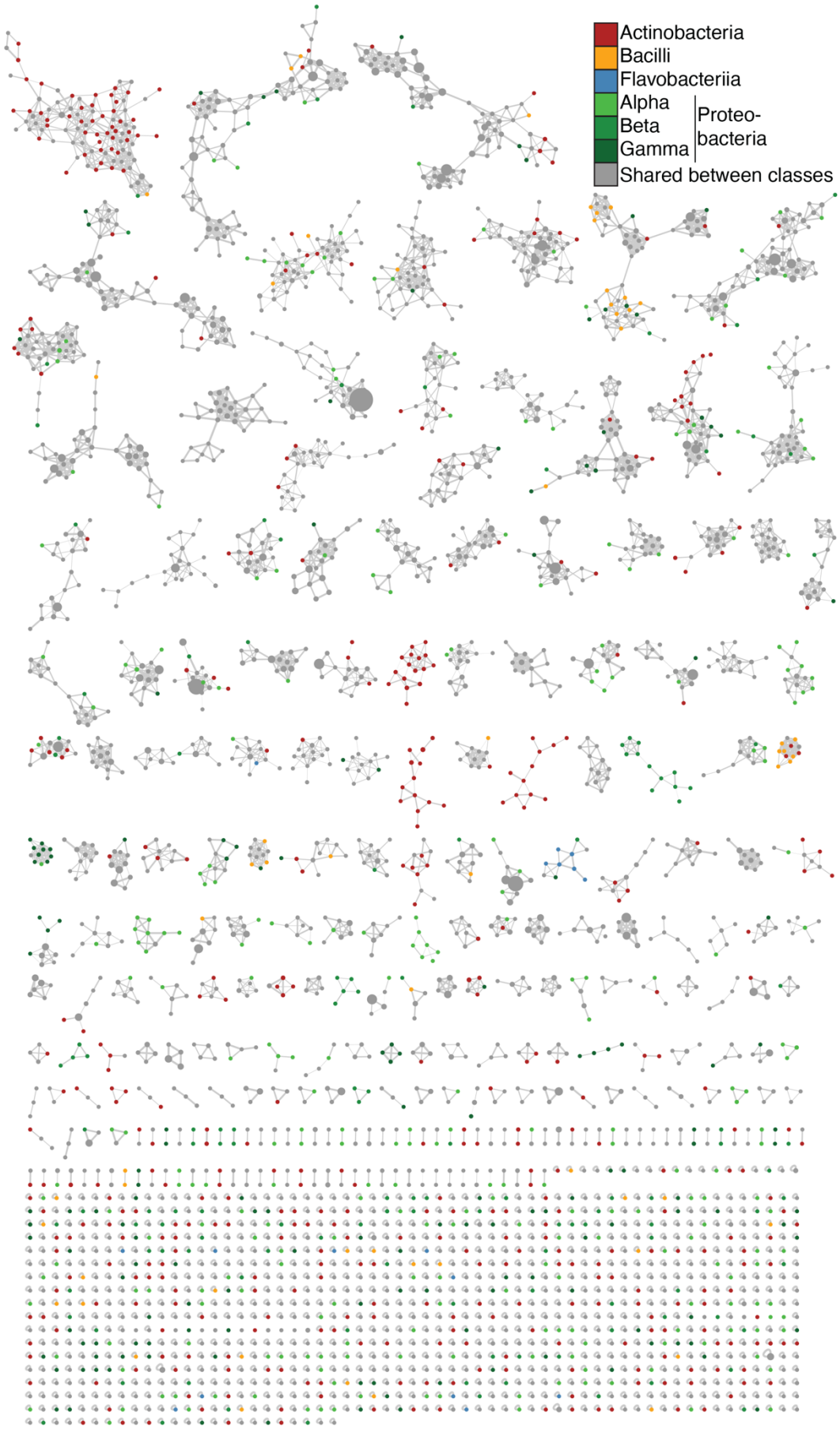


Figure S2 Identification of class-specific exometabolites

The metabolomes of all 198 individual strains were assessed using liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Molecular networking of bacterial metabolites obtained from organic extracts of the 198 strains, grown separately on 25% TSA for 96 h (Methods), was determined based on the Global Natural Products Social (GNPS) molecular networking workflow. The network consists of 3,314 nodes and 247 clusters (*i.e.*, molecular families) with at least two nodes and 1,046 singletons. Node sizes correspond to number of obtained spectra. Shared metabolites between at least two strains belonging to different classes are coloured in grey. Metabolites that are unique to one class are coloured according to the respective class colour code.

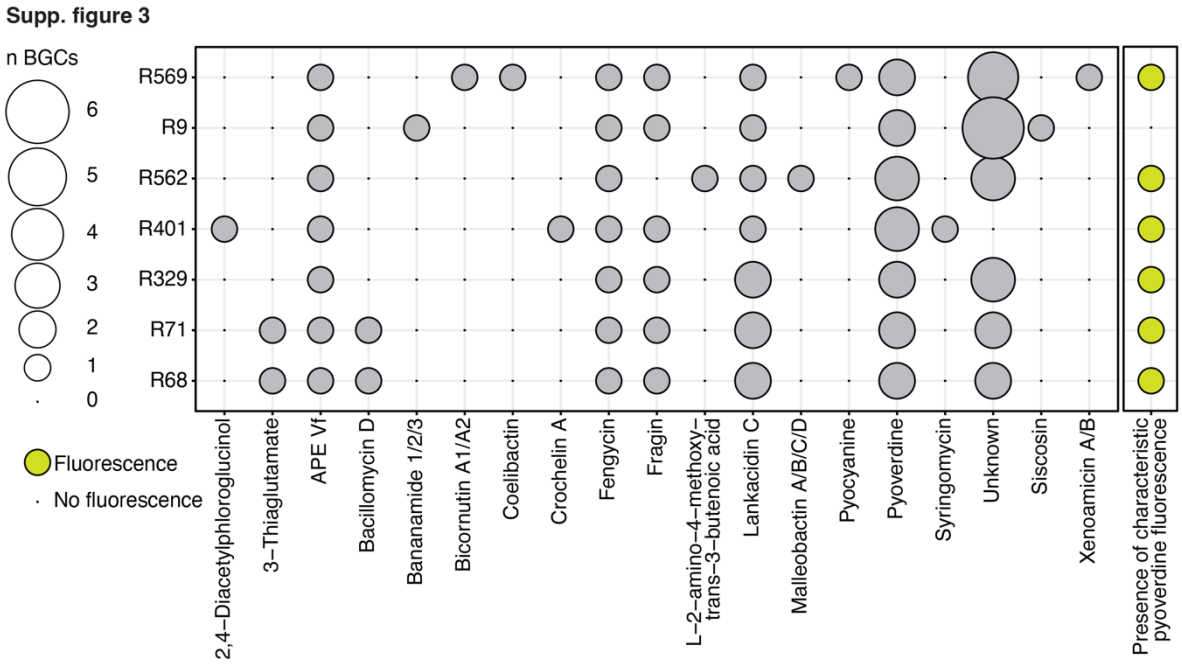
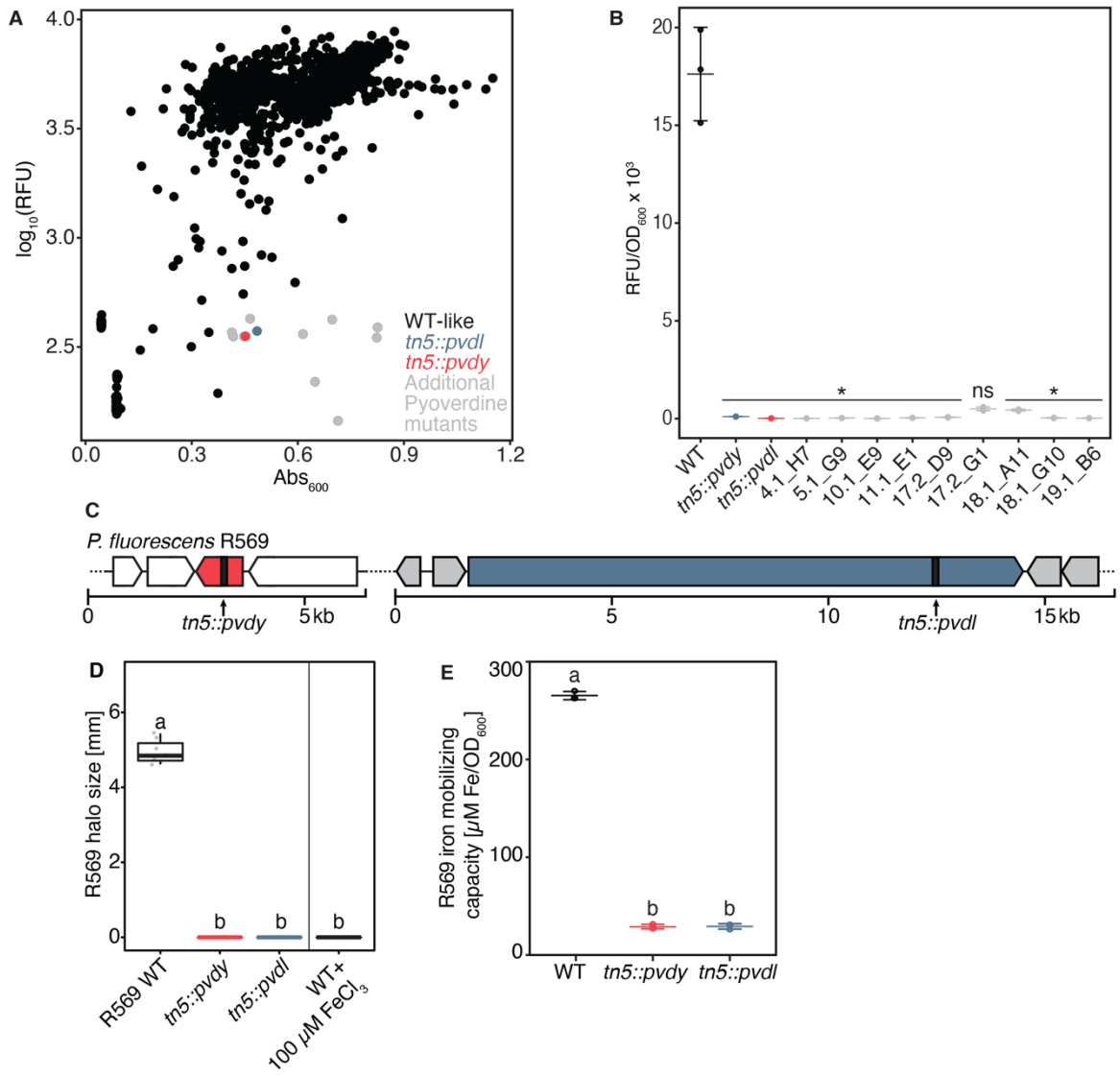


Figure S3 Genetic potential for the biosynthesis of specialized metabolites by all tested *Pseudomonas spp.* Strains

Using antiSMASH, we predicted BGCs for all genomes of the herein-included bacteria. All predicted BGCs within the seven tested *Pseudomonas spp.* strains are depicted in grey and are named according to the putatively produced metabolite. In light green, the presence or absence of pyoverdine-specific fluorescence, which is indicative of pyoverdine production, is highlighted.

Supp. figure 4



67

68 **Figure S4 Pyoverdine produced by *Pseudomonas fluorescence* R569 acts as a sole**
69 **inhibitory exometabolite**

70 (A) Initial screen for the lack of pyoverdine-specific fluorescence of approx. 2,000 R569
71 mutants with single random mini-Tn5 integration sites in the genome. Using a plate reader,
72 pyoverdine-specific fluorescence (excitation at 395 nm and emission at 470 nm) and OD_{600}
73 of individual R569 mutants were quantified. Twelve mutants with WT-like growth (> -1x
74 MAD) and reduced pyoverdine-specific fluorescence (< -6x MAD) are highlighted in blue,
75 red or light grey.

(B) Eleven out of the previously identified twelve putative pyoverdine mutants were independently confirmed for their lack of pyoverdine-specific fluorescence normalized to the mutants Abs_{600}. Statistical significance was determined by Kruskal-Wallis followed by Fisher's LSD post-hoc test and Benjamini-Hochberg adjustment. Significance compared to WT is indicated by black asterisks ($*$ indicate $p < 0.05$; n=3).

(C) Schematic overview of the genomic context of the fragmented *pvd*-operon which encodes a fraction of all pyoverdine biosynthetic genes of *P. fluorescence* R569. Genes within the BGC are coloured in grey, *pvdY* and *pvdL* are highlighted in red and blue, respectively. Transposon integration sites are highlighted by vertical black bars.

(D) Halo production of R569 WT and two transposon-insertion mutants that are impaired in the production of pyoverdine (t*n5::pvdy, tn5::pvdl)*. Mutant names and colours are depicted as in panel C. *Rs* was used as a target strain. Halo size measurements were taken after three days of interaction. Letters indicate statistically significant differences as determined by Kruskal-Wallis followed by Dunn's post-hoc test and Benjamini-Hochberg adjustment with $p < 0.05$ (n=9).

(E) Ferric iron mobilizing activity of R569 WT and mutants that are impaired in the production of pyoverdine. Letters indicate statistically significant differences as determined by Kruskal-Wallis followed by Dunn's post-hoc test and Benjamini-Hochberg adjustment with $p < 0.05$ (n=3).

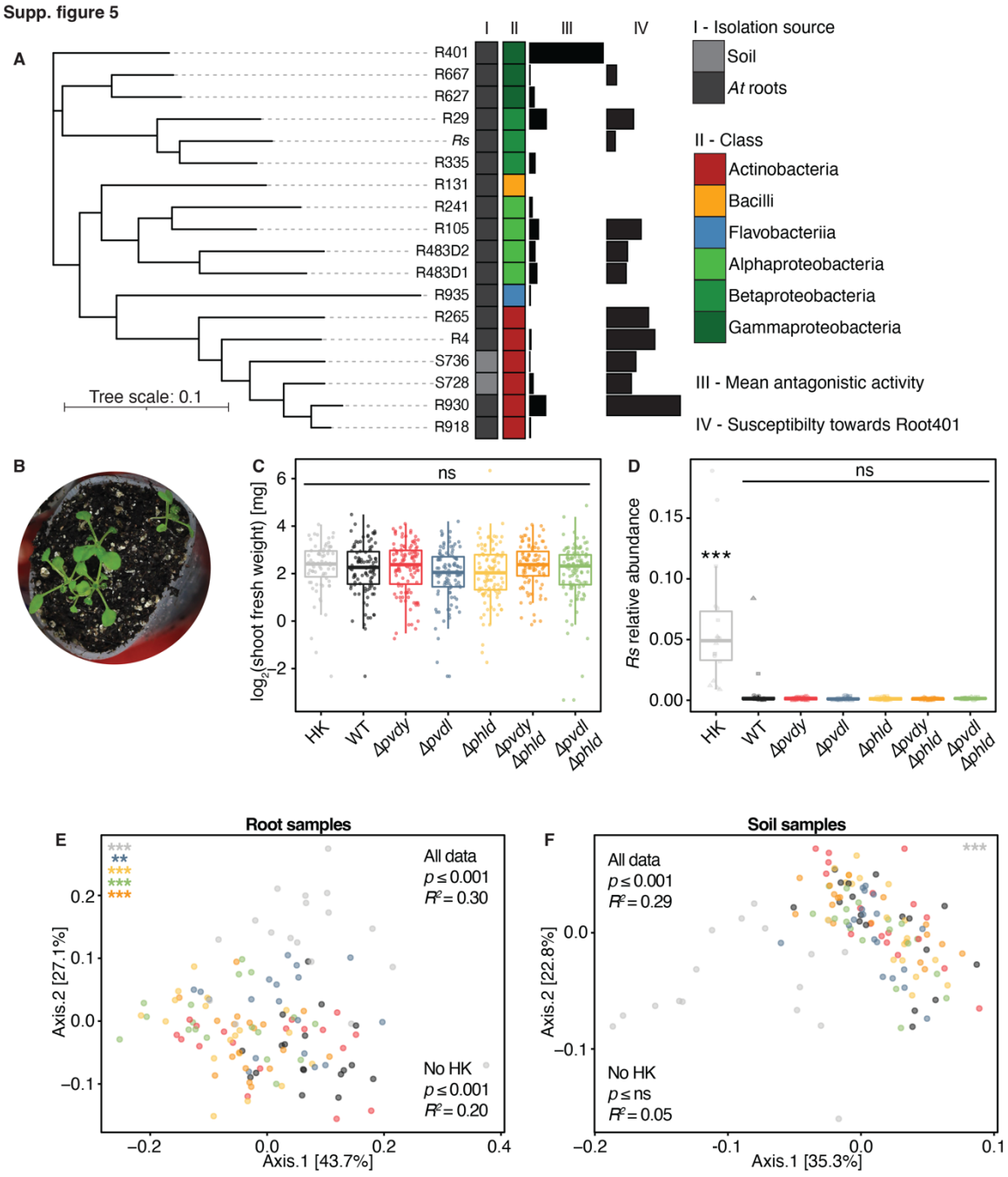
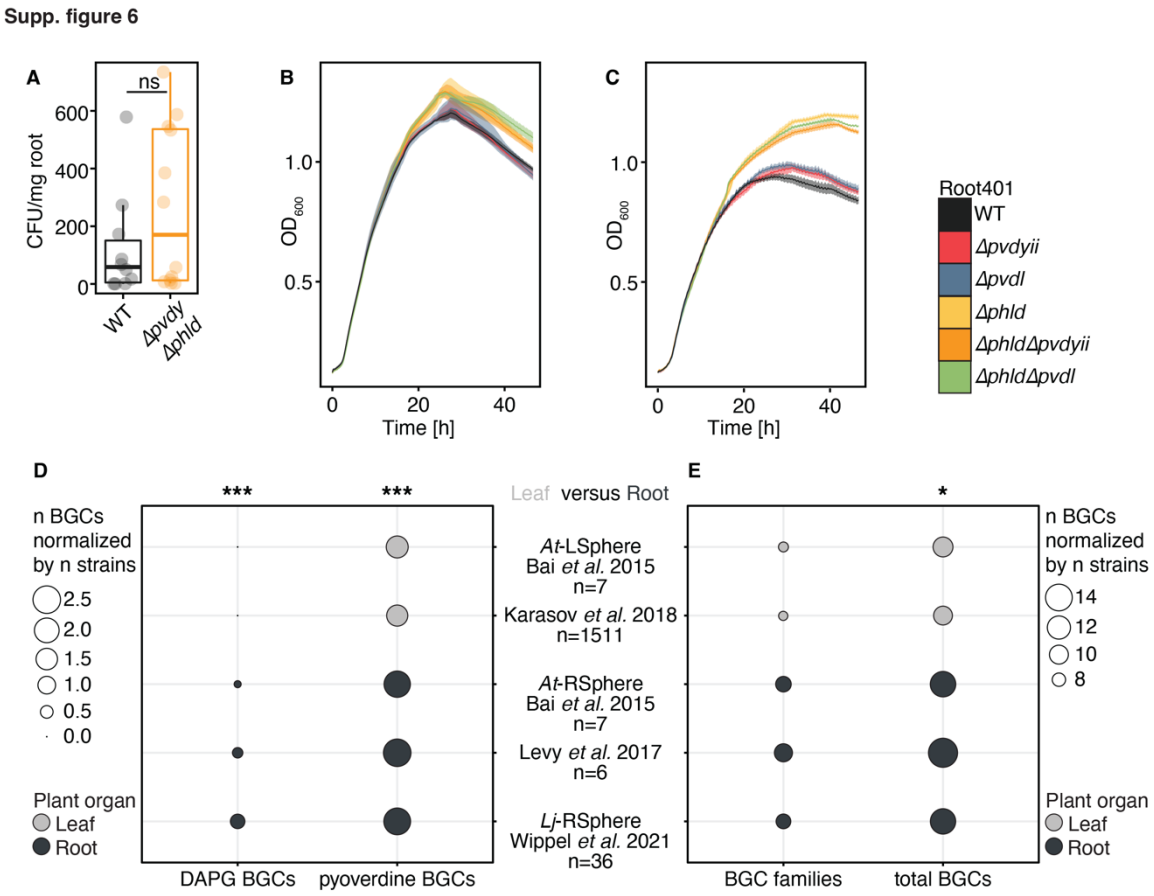


Figure S5 *R. solanacearum* does not cause disease symptoms in the tested conditions

(A) Phylogenetic tree of SynCom members based on v5v7 16S rRNA genes. The tree depicts the mean inhibitory activity (III) shown in Figure 1 as well as the mean sensitivity to R401 WT (IV) also derived from Figure 1. For more precise taxonomic assignments of SynCom members please see **Table S1**.

(B) Exemplary image of a Flowpot with HK treatment at 21 dpi.

(C) log2-transformed shoot fresh weight of *A. thaliana* plants grown in the gnotobiotic Flowpot system for 21 dpi. No statistical difference (ns) could be determined by Kruskal-Wallis followed by Dunn's post-hoc test and Benjamini-Hochberg adjustment (n=18).
(D) Relative abundance of *R. solanacearum* on the *A. thaliana* roots at 21 dpi. Statistical significance was determined by Kruskal-Wallis followed by Dunn's post-hoc test and Benjamini-Hochberg adjustment. Significance compared to WT is indicated by black asterisks (∗∗∗ indicates $p < 0.001$; ns, not significant; n=18).
(E–F) Unconstrained PCoA of bacterial beta diversity (Bray-Curtis dissimilarity) of root (E) and soil samples (F) in response to R401 or its mutants. Prior to computing relative abundances, R401 reads have been *in silico*-depleted to visualize solely the effect on the other SynCom members. PERMANOVA analysis-derived *p*-values are represented as asterisks (∗∗, ∗∗∗, indicate $p < 0.001$ or 0.001, respectively; n=18), coloured by the respective condition. PERMANOVA analysis on the full data set before (All data) or after (No HK) *in silico* depletion of HK samples are indicated in black; R^2 represents the variance explained by R401 genotype.



120

121 **Figure S6 R401 DAPG and pyoverdine BGCs are predominantly detected in the**
122 **genomes of root-derived *Pseudomonas sp.* isolates**

123 (A) Colonization efficiency as measured by colony forming units of R401 WT and the
124 Δ*pvdl*Δ*phld* double mutant on *A. thaliana* roots in mono-associations in the Flowpot system
125 at 21 dpi. No statistical difference (ns) could be determined by Kruskal-Wallis followed by
126 Dunn's post-hoc test and Benjamini-Hochberg adjustment (n=12).

127 (B–C) Growth curves of R401 WT and mutants in artificial root exudate (ARE) liquid
128 medium (B) and ARE without any addition of iron (C); n=6. Artificial root exudates are
129 used to recapitulate the nutritional status in the vicinity of the root.

130 (D–E) Using antiSMASH, we predicted BGCs for the genomes of *Pseudomonas sp.*
131 isolates from different root and leaf derived culture collections. For each culture collection,
132 the number of DAPG and pyoverdine BGCs (D) and the number of detected BGC families
133 and total BGCs (E) is depicted. Dot size indicates number of detected BGCs normalized

by the number of tested strains (n). Asterisks highlight statistical significance between root and leaf culture collections as measured by Chi-Square test (∗, ∗∗∗, indicate $p < 0.05$, and 0.001, respectively).

Supplementary tables

All supplementary tables have been deposited at Edmond:
https://doi.org/10.17617/3.6JTQY4

Table S1 mBA binary interaction data and SynCom selection.

Table S2 antiSMASH-based BGC predictions.

Table S3 Generated R401 and R569 mutants.

Table S4 Primers used in this study for amplification of the bacterial v5v7 *16S* rRNA region and for generation and validation of bacterial mutants.

Table S5 Artificial root exudates and siderophore assay media.

Material Availability

All generated bacterial mutant strains have been deposited in the bacterial culture collection of the Department of Plant Microbe Interactions at the Max Planck Institute for Plant Breeding Research in Cologne, Germany, and are available upon request from Stéphane Hacquard (hacquard@mpipz.mpg.de).

Data and Code Availability

Raw sequencing data from SynCom reconstitution experiments (MiSeq *16S* rRNA reads) has been deposited in the European Nucleotide Archive ENA at Accession: PRJEB56224. The circular PacBio-sequenced genome of R401 has been submitted to NCBI (BioProject: SUB12090952). The MS data of the comparative metabolomics are deposited and publicly available at GNPS as a MassIVE dataset at ftp://massive.ucsd.edu/MSV000081381. MS data of R401 are available at ftp://massive.ucsd.edu/MSV000090791/. Molecular networking jobs can be found under https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=91cec5b4f0074e5b868273a811295303 (198 strain network) and https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=511e72288abf4919b503e1784e183270 (interaction network). BGC predictions using antiSMASH can be found in **Table S2**. All supplementary tables have been deposited at Edmond: https://doi.org/10.17617/3.6JTQY4. All code and data files to reproduce the figures of this manuscript were deposited to https://github.com/scriptsFG/Getzke-Hassani-et-al.-2023.git.

Material and Methods

Bacterial Strains

The bacterial strains used in this study have been initially isolated from unplanted soil, *A. thaliana* roots or shoots (1) and are summarized in **Table S1**. *Ralstonia solanacearum* GMI1000 and GMI1600 have also been reported previously (2, 3). All mutants that were generated in the R401 or R569 backgrounds in this study have been deposited at the Max Planck Institute for Plant Breeding Research and are listed in **Table S3**.

Plant Growth Conditions

A. thaliana Col-0 wild-type (N60000) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). *Arabidopsis thaliana* Col-0 seeds were sterilized using 70% ethanol and bleach. Seeds were submerged in 70% ethanol and left shaking at 40 rpm for 14 minutes. Ethanol was removed before the seeds were submerged in 8.3% sodium hypochlorite (Roth) containing 1 µl of Tween 20 (Sigma-Aldrich) and left shaking at 40 rpm for 4 minutes. Under sterile conditions, the seeds were washed 7x times and finally taken up with sterile 10 mM $MgC1_2$. Seeds were left for stratification at 4 °C for 3 days. Seed sterility was confirmed by plating approx. 100 seeds on a 50% TSA plate.

Flowpot

Flowpots were assembled and inoculated as described below. Each Flowpot was first flushed with 50 ml sterile MiliQ water and then 50 ml half strength Murashige and Skoog medium with vitamins (½ MS; 2.2 g/l, Duchefa Biochemie, 0.5 g/l MES, pH 5.7) containing the bacterial inoculum. Per Flowpot, five surface-sterilized and stratified *A. thaliana* Col-0 seeds were pipetted. Microboxes were then incubated in a light cabinet under short day conditions (10 h light at 21 °C, 14 h dark at 19 °C) for 14 days and randomized every 2–3 days.

Agar-media

Surface-sterilized and stratified *A. thaliana* Col-0 seeds were sown on plates containing 1% agar (Bacto Agar, Difco) in ½ MS medium supplemented with 0.5% sucrose and placed vertically in a climate chamber (Panasonic, MLR-352) and grown for six days (10 h light,

21 °C; 14 h dark, 19 °C). Using a forceps, uniform seedlings were then transferred to freshly prepared ½ MS plates without sucrose.

Bacterial culture conditions

Bacteria were streaked from glycerol stocks (25% glycerol) on TSA plates (15 g/l Tryptic Soy Broth, Sigma Aldrich; with 10g/l Bacto Agar, Duchefa Biochemie) and grown at 25 °C. Single colonies were inoculated into liquid 50% TSB (15 g/l Tryptic Soy broth, Sigma Aldrich) and grown until dense at 25 °C with 180 rpm agitation. Dense cultures were then stored at 4 °C and diluted 1 to 10 in TSB the day before the experiment and cultured at 25 °C with 180 rpm agitation overnight to ensure sufficient cell densities for slow- and rapidly growing bacteria. Glycerol stocks were stored at -80 °C and kept on dry ice when transported.

Screen for antagonistic interbacterial interactions

For the initial mBA experiment (**Fig. 1**), bacterial strains were cultured for seven days in 25% TSB (7.5 g/l Tryptic Soy Broth, Sigma-Aldrich). Briefly, 100 µl of a bacterial solution were re-suspended in 50 ml cooled (~38 ºC), but still molten, 25% TSA (15 g/l Bacto-Agar, Duchefa Biochemie) and poured into a square petri dish (120x120 mm). After medium solidification, 24 bacterial isolates were spotted on top of the medium using a multi-stamp replicator. The replicator was sterilized by dipping in 70% EtOH (v/v) followed by flaming and cooling. The screen comprising 39,204 binary interactions was conducted once and validated by randomly re-screening 7,470 interaction pairs as described above. All bacterial strains that showed antagonistic activity were re-screened two more times. For the *Ralstonia* inhibition screen, *R. solanacearum* GMI1000 was pre-cultured for two days in CPG medium (1% peptone, 0.5% glucose and 0.1% casamino acids; pH7.0). Before spotting the bacterial cultures, each CPG agar plate (1% Peptone, 0.5% D-glucose, 0.1% casamino acids and 1.5% agar; pH 7.0) was overlaid with 5 ml of *R. solanacearum* suspension (50 µl of pre-cultured *R. solanacearum* in pure sterile water). Excess *R. solanacearum* suspension was removed and the plates were briefly dried, then 5 µl of the bacterial culture were spotted onto the *R. solanacearum*-overlaid plates. All isolates were tested three times. For all subsequent halo assays, strains were cultivated in 50% TSB until

turbidity, stored at 4 °C and diluted 1:10 in 50% TSB one day before the experiment. Bacterial cultures were pelleted at 4,000 rpm for 15 min. The resulting bacterial pellets were subsequently washed 3 times and resuspended in 1 ml 10 mM $MgCl_2$. OD_{600} were measured and set depending on the strain. One hundred microliters bacterial culture were inoculated per 50 ml 25% TSA. After drying, up to nine different 3-µl droplets of bacterial suspensions with 0.4 OD_{600} were applied with equal distances. For all experiments, plates were incubated at 25 °C for up to 96 hours and photographs were taken thereafter for quantitative image analysis. The size of the halo of inhibition was measured using ImageJ with up to five separate measurements, which were subsequently averaged to reduce variation. Raw data of Fig. 1 are indicated in **Table 2**.

Metabolomic Analyses

Metabolites were extracted from individual isolates (n = 198) grown on the same agar medium used in mBA experiments with two organic solvents with different polarity, ethyl acetate and methanol, to capture greater small molecule diversity. Each bacterial strain was grown separately on 25% TSA plates (25% BBL™ Trypticase™ Soy, BD with 1.8% Bacto-Agar; BD, Germany). After seven days of incubation at 25 °C, three to four agar plugs were taken from the periphery and inside of the bacterial colony. Agar plugs were crushed and washed with 500 µl water followed by extraction in 500 µl ethyl acetate and methanol. Between each extraction step, samples were vortexed for 30–45 s. After each extraction, the solvents were evaporated, and the residue was redissolved in 500 µl LC-MS-grade methanol and filtered through a 0.2-µm membrane into HPLC vials. Solvents for blanks (uninoculated medium) were extracted according to the same protocol. The extraction protocol was also used to analyse the inhibition zones upon inter-bacterial interactions. To this end, a bacterial lawn of a sensitive target strain, either R472D3, R480 or R553, was prepared as top agar and the antibiotic producer strains (R63, R68, R71, R342, R401, R562, R569, R690, R920 and R1310, respectively) were inoculated on top. The agar plugs were taken from the zone of inhibition and inside of the antibiotic-producing colony. In total, 20 interactions were analysed. All samples were analysed by HPLC-MS/MS on a micrOTOF-Q mass spectrometer (Bruker) with ESI-source coupled

with a HPLC Dionex Ultimate 3000 (Thermo Scientific, Germany) using a Zorbax Eclipse Plus C18 1.8 µm column, 2.1×50 mm (Agilent). The column temperature was 45 °C. MS data were acquired over a range 100–3000 m/z in positive mode. Auto MS/MS fragmentation was achieved with rising collision energy (35–50 keV over a gradient from 500–2000 m/z) with a frequency of 4 Hz for all ions over a threshold of 100. uHPLC began with 90% H_2O containing 0.1% acetic acid. The gradient started after 0.5 min to 100% acetonitrile (0.1% acetic acid) in 4 min. Two microliters sample solution were injected to a flow of 0.8 ml/min. All MS/MS data were converted to '.mzxml' format and transferred to the GNPS server (gnps.ucsd.edu) (4). Molecular networking was performed based on the GNPS data analysis workflow using the spectral clustering algorithm (5). The data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor m/z. MS/MS spectra were window-filtered by choosing only the top 6 peaks in the +/- 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. Consensus spectra that contained less than two spectra were discarded. Networks were then created from the single cultivation and from the competition experiments. Edges were filtered to have a cosine score above 0.5 (0.6 for interaction network) and more than four matched peaks. Further edges between two nodes were kept in the network only if each of the nodes appeared in each other's respective top ten most similar nodes. Sample attributes were assigned to the data files (strain, genus, family, order, class phylum extraction solvent). For the network analysis, all nodes that contained ions from the blank medium were removed. The network was visualized using Cytoscape 3.5.1.

Detection of R401 DAPG and pyoverdine

For the detection of iron-chelating compounds, a R401 preculture was grown over night in 5 ml LB medium at 30 °C and 200 rpm. Before inoculation of the main culture, cells were washed twice with the main culture medium to remove potential traces of iron from the medium. Erlenmeyer flasks containing 20 ml of modified MM63 (KH_2PO_4 13.61 g/L KOH 4.21 g/L, $(NH_4)SO_4$ 1.98 g/L, $MgSO_4$*7 H_2O 0.25 g/L, NaCl 0.5g/L, Glucose*H_2O 5,00 g/L, pH 7.1 KOH/HCl) with or without addition of 0.0011 g/L $FeSO_4$*7 H_2O were

inoculated with 100 µl of preculture and cultivated for 6 days at 30 °C and 200 rpm. Every second day, 0.5 ml sample were taken, cells were removed by centrifugation and 5 µl of supernatant were analysed on a Bruker microTOFq-II high-resolution mass spectrometer coupled to an Agilent 1290 UPLC system with an Acquity UPLC BEH C-18 reverse phase column, run in a gradient of MeCN/H2O + 0.1% formic acid. Higher accuracy measurements were performed on a maXis-II qTOF, coupled to an identical LC setup as described earlier.

PacBio sequencing and assembly

PacBio-sequenced reads were obtained from Max Planck Genome Centre (https://mpgc.mpipz.mpg.de). PacBio 2kb sequence control reads were removed with blasr (6). Reads were assembled using Flye v2.8-b1673 (7) and polished four times using the internal function. Then, assembly went through a final polishing step with Medaka v1.0.3 (Oxford Nanopore Technologies Ltd, 2018). Annotation was conducted using Prokka 1.12-beta (8), and output files (.ffn, .faa, .gff) were parsed with a custom Python script. Then, we examined; (I) statistics of assemblies and visualisation of assemblies with Bandage v0.8.1 (9); (II) integrity of assemblies using BUSCO v4.0.6 (10) with bacteria_odb10 (https://busco-data.ezlab.org/v4/data/lineages/); and (III) potential contamination with other species with Blobtools2 v2.2 (11). Phylogenetic assignment of R401 as *Pseudomonas brassicacearum* is based whole genome phylogeny.

BGC prediction using antiSMASH

Bacterial genomes were downloaded from "www.at-sphere.com" (1) or NCBI and submitted to https://antismash.secondarymetabolites.org/ version 6.0 (12). Output data from antiSMASH analysis are listed as BGC classes and predicted BGCs for each genome in **Table S2**. Only high-quality genomes, as assessed by CheckM with ≥90% completeness and ≤5% contamination ratio were used for the analysis. For R401, the PacBio-sequenced high-quality genome was used for BGC prediction using antiSMASH.

Mutant generation

Marker-free knockouts in R401 were generated through homologous recombination using the cloning vector pK18mobsacB (GenBank accession: FJ437239), which encodes the *kanR* and *sacB* genes conferring resistance to kanamycin and susceptibility to sucrose, respectively. In this method, upstream and downstream sequences of the gene to be deleted are integrated into the pKl8mobsacB suicide plasmid by Gibson assembly (13). The resulting plasmid is transformed into BW29427 *E. coli* cells and subsequently conjugated into R401. The plasmid is then integrated into the chromosome by homologous recombination and deletion mutants are generated by a second sucrose counter-selection-mediated homologous recombination event. The protocol is adapted from (14).

Generation of pK18mobsaB-derived plasmid containing flanking regions of the gene of interest.

Primers were designed to amplify a 750-bp DNA sequence (*i.e.*, flanking region) directly upstream and downstream of the target region, sharing terminal sequence overlaps to the linearized pK18mobsacB vector and the other respective flanking region using Geneious Prime. R401 genomic DNA was isolated from 6 µl dense R401 culture in 10 µl of buffer I (pH 12) containing 25 mM NaOH, 0.2 mM EDTA at 95 °C for 30 min, before the pH was readjusted using 10 µl of buffer II (pH 7.5) containing 40 mM Tris-HCl. The R401 genomic DNA was used for amplification of the flanking regions through PCR using the respective flanking region-specific primer combinations (**Table S4**). PCR was conducted with 0.2 µl Phusion Hot Start High-Fidelity DNA polymerase (New England Biolabs) in 20-µl reactions containing 4 µl 5x Phusion HF buffer (New England Biolabs), 0.4 µl 10 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 2 µl of R401 genomic DNA as template, filled up to 20 µ1 with nuclease-free water. The tubes were placed into a preheated (98 °C) thermal cycler set at the following program: 98 °C for 30 s, 35 cycles of 98 °C for 7 s, 60 °C for 20 s, 72 °C for 15 s, then a final extension at 72 °C for 7 min. Five microliters of the PCR product were combined with 1 µl Orange DNA Loading Dye (6x; New England Biolabs), loaded on 1% agarose gels containing 0.05% EtBr, and run at 110 mV. After confirmation of successful amplification, the PCR product was purified

using AMPure XP (Beckman-Coulter) and subsequently quantified using Nanodrop (Thermo Fisher Scientific). Plasmid purification was performed on an *E. coli* culture containing plasmid pK18mobsacB using the QIAprep Spin Miniprep Kit for plasmid DNA purification (QIAGEN) following the manufacturer's instructions. The pkl8mobsacB vector was then amplified and linearized through PCR using the PKSF and PKSR primers (**Table S4**). PCR was conducted with 0.2 µl Phusion Hot Start High-Fidelity DNA polymerase (New England Biolabs) in 20-µl reactions, largely as described above with 1 µl 0.1 ng/µl pkl8mobsac as a template. Annealing temperature was decreased to 55 °C and extension time increased to 150 s for each cycle. Template DNA was digested by DpnI (New England Biolabs) in 50-µl reactions containing 1 µl DpnI, 1 µg DNA, 5 µl Cutsmart buffer (New England Biolabs) and filled up to 50 µl with nuclease-free water. The tubes were then incubated at 37 °C for 15 min followed by heat inactivation at 80 °C for 20 min. Five microliters of the DpnI-digested plasmid were combined with 1 µl Orange DNA Loading Dye and analysed by DNA agarose electrophoresis. Upon successful verification of amplification and digestion, the remaining sample was purified using AMPure XP and subsequently quantified using Nanodrop. Linearized pK18mobsacB and both flanking regions were mixed in a molar ratio of 1:3:3 into a 10-µl total volume, added to 10 µl 2X Gibson Assembly® Master Mix (New England Biolabs) and incubated at 50 °C for 1 h.

Transformation into chemically competent E. coli BW29427 cells

The vector was transformed into 50 µl chemically competent BW29427 *E. coli* cells according to the following heat shock protocol: 2 µl of the vector were gently mixed with 50 µl of competent cells, and the resulting mixture was incubated on ice for 30 min. The mixture was transferred to a water bath at 42 °C for 1 min and put back on ice for 2 min. Then, 1 ml of 50% TSB with 50 µg/ml diaminopimelic acid (DAP; Sigma-Aldrich) was added to the heat-shocked cells, the mixture was left to regenerate at 37 °C for 1 h and then plated on 50% TSA containing 25 µg/ml Kanamycin (Kan) and 50 µg/ml DAP. The plates were incubated at 37 °C overnight. Resulting colonies were validated by colony PCR using the M13F and M13R primers. Colony PCR was performed on at least four separate colonies with 0.4 µl DFS-Taq polymerase (BIORON) in 25 µl reactions containing 2.5 µl l0x

386 incomplete buffer (BIORON), 0.5 10 mM MgC12, 0.5 µl 10 mM dNTPs, 0.75 µl 10 µM
387 forward primer, 0.75 µl 10 µM reverse primer, a small fraction of a colony and filled up to
388 25 µl with nuclease-free water. The tubes were placed in a thermocycler set at the following
389 program: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, then
390 a final extension at 72 °C for 10 min. Five microliters of the PCR product were combined
391 with 1 µl Orange DNA Loading Dye and analysed by DNA agarose electrophoresis.
392 Positive colonies were purified by streaking on new 50% TSA plates containing 25 µg/ml
393 Kan and 50 µg/ml DAP and further verified by Sanger sequencing (Eurofins Scientific)
394 following the manufacturer's protocol.

395

396 *Conjugation of E. coli and R401 and selection for first homologous recombination*
397 *event*

398 *E. coli* BW29427 cells containing the plasmid and R401 were inoculated into 4 ml of 50%
399 TSB containing 25 µg/ml Kan and 50 µg/ml DAP or 50% TSB and incubated overnight at
400 37 °C with 180 rpm agitation or 25 °C with 180 rpm agitation, respectively. Cells were
401 harvested by centrifugation at 8,000 rpm for 2 min at room temperature, then washed 3x
402 and subsequently resuspended in 1 ml of 50% TSB followed by centrifugation, after which
403 the supernatant was discarded. After quantifying OD_{600}, both cultures were mixed to equal
404 parts and approx. 10x concentrated by centrifugation. The bacterial suspension was plated
405 on 50% TSA plates containing 50 µg/ml DAP and incubated at 25 °C overnight to allow
406 for conjugation events. The mating patches were scraped of the plate and resuspended in 1
407 ml 50% TSB. Then, 100 µl were spread on 50% TSA plates containing 25 µg/ml Kan and
408 50 µg/ml Nitrofurantoin (Nitro; Sigma-Aldrich; to counter-select *E. coli*) and incubated at
409 25 °C. Colonies were validated for successful genomic insertion of the plasmid *via* colony
410 PCR using a primer specific to the genomic DNA approx. 150 bp upstream of the upward
411 flanking region (upup) and the plasmid specific M13F primer. Colony PCR was performed
412 on at least 15 separate colonies and a WT control with 0.4 µl DFS-Taq polymerase in 25-
413 µl reactions as described previously, but with an annealing temperature of 60 °C. Five
414 microliters of the PCR product were combined with 1 ml Orange DNA Loading Dye and

415 analysed by DNA agarose electrophoresis followed by Sanger sequencing following the
416 manufacturer's protocol.

417

418 *Sucrose counter-selection to induce the second homologous recombination event*

419 A R401 colony with a successful genomic insertion of the plasmid was resuspended from
420 a plate into 1 ml of 50% TSB. The cell density in the medium was then measured using the
421 Multisizer 4e Coulter Counter (Beckman Coulter) following the manufacturer's protocol.
422 One hundred microliters of 500 cells/µl, 5,000 cells/µl and 50,000 cells/µl dilutions were
423 spread on three separate 50% TSA plates containing 300 mM sucrose. The plates were
424 incubated at 25 °C for approx. 48 h. At least 30 colonies were examined by colony PCR
425 using the respective upup and dwdw primers. Colony PCR was performed with 0.4 µl DFS-
426 Taq polymerase in 25-µl reactions as described previously with an annealing temperature
427 of 60 °C. Five microliters of the PCR product were combined with 1 µl Orange DNA
428 Loading Dye and analysed by DNA agarose electrophoresis. Positive colonies were
429 purified by streaking on new 50% TSA plates and further verified by Sanger sequencing
430 (Eurofins Scientific) following the manufacturer's protocol. They were also streaked on
431 50% TSA containing 25 µg/ml Kan to verify loss of the plasmid. A second colony PCR
432 was performed on positive colonies and a wt control to validate the absence of the GOI,
433 using a forward (inF) and reverse (inR) primer inside the GOI. Colony PCR was performed
434 with 0.4 µl DFS-Taq polymerase in 25 µl reactions as described previously. Five
435 microliters of the PCR product were combined with 1 ml Orange DNA Loading Dye and
436 analysed by DNA agarose electrophoresis. Upon successful verification, 4 ml of 50% TSB
437 were inoculated with a positive colony and grown overnight at 25 °C at 180 rpm. Finally,
438 750 µl of the overnight culture were added to 750 µl of 50% glycerol in an internally
439 threaded 1.8 ml Nunc CryoTube, gently mixed, and stored at -80 °C.

440

441 *Establishment of mini-Tn5 transposon mutant collections in R401 and R569*
442 Mini Tn5-mutant collections of R401 and R569 were established similarly with only minor
443 changes as described below. Liquid cultures of R401 or R569 and *E. coli* strain BW29427

carrying plasmid pUTmTn5Km2 (15) were grown overnight in no antibiotics or 25 µg/ml Kan and 50 µg/ml DAP at 25 °C or 37 °C, respectively. Conjugation was carried out as described above in "*Conjugation of E. coli and R401 and selection for first homologous recombination event"*. For R401, the mating patch was taken up in 1 ml 50% TSB liquid medium and subsequently plated on 50% TSA plates containing 25 µg/ml Kan and 50 µg/ml Zeocin in four different dilutions (undiluted, 1:3, 1:4, and 1:5) and left to grow at 25 °C for 48 h. Individual colonies were picked in 100 µl sterile 50% TSB in 96-well culture plates, sealed and left to grow at 25 °C and 180 rpm for 24 h. Subsequently, 100 µl 50% glycerol were added to each well and plates were frozen until further processing. The outer rows and columns were left uninoculated as to avoid positional effects in the subsequent forward genetic screen. For R569, resuspended mating patches were stocked at -80 °C in 700-µl aliquots using a final concentration of 25% Glycerol. 1:4 dilutions were plated onto 50% TSA plates supplemented with 120 µg/ml Kan, 50 µg/ml Rifampicin and 50 µg/ml Zeocin and incubated at 25 °C for 48h. Individual colonies were inoculated in 100 µl 50% TSB supplemented with the same antibiotics at the same concentrations in 96-well plates and incubated at 25 °C and 180 rpm for 48 h. Then, 100 µl 50% glycerol were added to each culture and plates were frozen at -80 °C.

Mini-Tn5 transposon mutant screen for loss of R401s growth inhibition of Rs GMI1600

Each R401 mini-Tn5 transposon mutant was screened individually for loss of inhibitory activity against GFP-expressing *Rs* GMI1600 and for wild type-like growth, as we observed in first trials that mutants that are impaired in growth are also more likely to have reduced inhibitory activity against *Rs* GMI1600. This screen was conducted in a 96-well plate format with GFP expression of *Rs* GMI1600 in response to individual R401 mutants and Abs_{600} of axenically grown, individual R401 mutants as readouts. Therefore, for each mutant, two wells were inoculated in parallel, one in the presence of *Rs* GMI1600 and one grown axenically. *Rs* GMI1600 was streaked on 50% TSA plates and left to grow at 25 °C for 96 h. The same day, R401 mutants were each inoculated into 150 µl Artificial Root Exudates (ARE; **Table S5**) in 96-well culture plates ('R401 preculture plates') from glycerol stocks using a multi-stamp replicator and left to grow at 25 °C and 150 rpm for 96

h until saturation. Then, a *Rs* GMI1600 preculture was inoculated into 10 ml ARE and grown overnight at 25 °C and 150 rpm. Approximately 24 h later, the preculture was 1:10 diluted with 90 ml ARE and left to grow under the same conditions. Approximately 24 h later, the 100 ml *Rs* GMI1600 culture was concentrated by centrifugation at 4,000 rpm for 15 min, washed 3x and resuspended in ARE. Subsequently, OD_{600} was quantified and set to 0.2 in ARE. Then, 75 µl of this *Rs* GMI1600 suspension were transferred to each well of a sterile 96-well bacterial culture plate (Greiner-CELLSTAR-96-Well plate, transparent, flatbottom; Sigma-Aldrich) per 'R401 preculture plate', referred to as the 'R401-*Rs* interaction plate'. Per 'R401 preculture plate' one sterile 96-well bacterial culture plate (Greiner-CELLSTAR-96-Well plate, transparent, flatbottom; Sigma-Aldrich) was filled with 150 µl ARE per well and R401 mutants were inoculated from the 'R401 preculture plate' using a multi-stamp replicator; this plate is referred to as the 'axenic R401 plate'. After mixing by pipetting, 75 µl R401 mutant suspension were transferred from the 'axenic R401 plate' to the 'R401-*Rs* interaction plate'. Finally, 75 µl ARE were added to each well of the 'axenic R401 plate'. The 'axenic R401 plate' and 'R401-*Rs* interaction plate' thereby contain the same volume and concentration of R401 mutants, while the latter also contains a final concentration of OD_{600} 0.1 *Rs* GMI1600 per well. Both plates were closed with a lid and incubated at 25 °C and 150 rpm for 48 h. Subsequently, Abs_{600} was measured for the 'axenic R401 plate' and GFP fluorescence was quantified at $\lambda_{excitation}$=475 nm and $\lambda_{emission}$=510 nm for the 'R401-*Rs* interaction plate'. Both measurements were taken using a microplate reader (Infinite M200 PRO, Tecan). Subsequently, candidate R401 mutants were selected based on two criteria: (I) loss of *Rs* GMI1600 inhibition; a mutant was considered a candidate if the GFP fluorescence in the 'R401-*Rs* interaction plate' was lower than 3-fold the absolute deviation around the median (MAD) (16) compared to the respective plates median, and (II) wild-type-like growth; a mutant was considered a candidate if the Abs_{600} in the 'axenic R401 plate' was not lower than 3x MAD compared to the respective plate's median. Candidate R401 mutants were freshly picked from glycerol stocks and validated twice more independently using the same assay. Finally, 38 mutants that showed wild-type-like growth and robustly reduced inhibitory activity against *Rs* GMI1600 were subsequently tested in an orthogonal mBA experiment with *Rs* as target bacterium, as described before.

Mini-Tn5 transposon mutant screen for lack of pyoverdine fluorescence of R569

R569 mini-Tn5 mutants were cultured in 96 well plates at 25 °C and 180 rpm for five days in 50% TSB medium. For the initial mutant screen, fluorescence was acquired at $\lambda_{excitation}$=395 nm and $\lambda_{emission}$=470 nm in a microplate reader (Infinite M200 PRO, Tecan). Out of ~2,000 mutants analysed, the fluorescence-based screening identified twelve R569 mutants that showed severely reduced pyoverdine-specific fluorescence but retained median-like growth behaviour (Thresholds: -6x MAD fluorescence, >-1x MAD Abs_{600}). For validation, cultures were pre-grown for five days in 50% TSB before sub-culturing into fresh siderophore medium (see **Table S5**) and growth for five additional days. Finally, bacterial culture density (Abs_{600}) was determined, and pyoverdine-specific fluorescence of bacterial culture supernatants was captured at $\lambda_{excitation}$=410 nm and $\lambda_{emission}$=500 nm. For comparison between genotypes, fluorescence capacity was calculated by dividing the pyoverdine-specific fluorescence by culture density.

Identification of mini-Tn5 transposon integration sites in the genomes of R401 and R569

The chromosomal mini-Tn5 transposon integration sites in the R401 or R569 genomes were determined similarly as described before (15). Briefly, a colony from each strain was resuspended in 20 µl of sterile deionized water. Subsequently, a two-step PCR (TAIL-PCR) was performed starting with an arbitrarily primed PCR (PCR1), followed by a nested PCR (PCR2) on the generated product using the primers as described (**Table S4**). PCR reactions were conducted using the BIORON DFS-Taq polymerase reaction kit according to the manufacturer's instructions. One microliter of a resuspended bacterial colony was used as template for PCR1 in a total PCR reaction volume of 25 µl. PCR1 was conducted using primers JO4 and JO28 and the following steps were applied: 95 °C for 5 min, six cycles of 95 °C for 15 sec, 30 °C for 30 sec and 1 min elongation at 72 °C, followed by 30 cycles with 95 °C for 15 seconds, 45 °C for 30 seconds and 1 min elongation at 72 °C. Final elongation was for 5 min at 72°C. PCR2 was conducted using 0.3 µl of the PCR1 product and the primers JO1 and JO5. The following steps were applied: 95 °C for 5 min followed by 30 cycles of 95 °C for 15 sec, 57 °C for 30 sec and 1 min elongation at 72 °C. Final elongation was for 5 min at 72°C. R401 or R569 wild type was included to account

for unspecific amplifications. PCR products were separated by agarose gel electrophoresis. One of the most prominent bands from each sample was extracted using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel). The PCR product was eluted into a final buffer volume of 20 µl; DNA concentration was determined with a spectrophotometer (NanoDrop One, Thermo Scientific), and 75 ng of each product were sent for Sanger sequencing (Eurofins genomics). Finally, chromosomal Tn5 integration sites were assessed by alignment of the obtained sequences flanked by the integrated Tn5 transposon in the R401 or R569 genome using the Geneious Prime or CLC Genomics Workbench software, respectively (Qiagen Digital Insights). Matching open reading frames (ORFs) were further aligned to the NCBI BLAST (17) nucleotide and protein data base (using BLASTn and BLASTp algorithm).

Complementation of R401 Δpvdy

Complementation of R401 Δ*pvdy* was conducted by expressing the coding region of R401 *pvdY* under its putative native promoter from a low-copy plasmid in the Δ*pvdy* mutant background. Plasmid construction was conducted using Gibson assembly as described before. In brief, the coding region of R401 *pvdY* was co amplified with a 1.5 kb region upstream of the gene and ligated into a linearized pSEVA22l (18) vector backbone. This vector was integrated into *E. coli* BW29427 cells and subsequently integrated into R401 Δ*pvdy via* bi-parental conjugation.

In vitro iron mobilization assay

The capability of R401 and R569 mutants of solubilizing inaccessible ferric iron was tested using a previously described photometric assay (19). Bacteria were cultured at 25 °C and 180 rpm for five days in 50% TSB and subsequently subcultured and 1:50 diluted in siderophore medium (**Table S5**). Diluted cultures were grown for an additional five days under the same conditions. At five days post inoculation (dpi), bacterial load was quantified by OD_{600} determination. Subsequently, cells were pelleted by centrifugation for 15 min at 4,000 rpm. The cell-free supernatant was diluted 5-fold with 10 mM $MgCl_2$ and 25 µl were mixed with 100 µl of CAS assay solution (20) in three technical replicates and incubated

for approx. 40 min at room temperature in the dark. Using an Infinite 200 PRO plate reader (TECAN), Abs_{636} was measured as an indication for the transition from complexed iron (blue complex) to solubilized or siderophore-bound iron (yellow). Finally, bacterial iron mobilizing capacity was computed according to the following formula:

$$Fe\text{–}mobilizing\ capacity\left[\frac{\mu M\ Fe}{OD_{600}}\right] = \frac{\left(\frac{(Abs_{636}(Medium) - Abs_{636}(Sample))}{(Abs_{636}(Medium)}\right) * 2\ nmol}{(Volume(Supernatant) * OD_{600})}$$

Validation of bacterial growth rates

The growth of individual R401 mutants and wild type was assessed by continuously measuring OD_{600} of actively growing bacterial cultures in an Infinite 200 PRO plate reader (TECAN) over 48 h at 25 °C and approx. 300 rpm. Overnight cultures in 50% TSB were pelleted and washed as described before. Subsequently, OD_{600} was measured and set to 0.02 in either artificial root exudates (ARE) or ARE lacking $FeCl_3$. Composition of ARE was adopted from (21) and can be found in **Table S5**.

SynCom selection

The 18-member SynCom utilized in our study was designed to allow for strain-specific tracking of each strain based on the bacterial v5v7 *16S* rRNA region while incorporating a taxonomically diverse set of strains spanning all core classes that stably associate with Arabidopsis roots across a European transect, as well as soil-derived strains (22). Furthermore, we aimed to represent strains that were either sensitive or non-sensitive to R401 based on mBA experiments approximately at a ratio that is representative at least for the tested strains in the At-Sphere. The size of the 18-member SynCom was based on prior experiences, as similarly sized communities were shown to be easily tractable while at the same time sufficiently complex to recapitulate host preference in the root microbiota of Arabidopsis and Lotus in natural soils, an important community-level microbiota trait (23).

Microbiota reconstitution in the gnotobiotic Flowpot system

Flowpot assembly was performed according to (24) with minor adjustments. A 2:1 mixture of peat potting mix and vermiculite was used as a matrix. The matrix was sterilized two times (25 min liquid cycle (121 °C) and 45 min solid cycle (134 °C)) and stored at 60 °C until completely dry. Prior to Flowpot assembly, the matrix was rehydrated with sterile MiliQ water. Flowpots were assembled by adding a layer of glass beads to the conical end of a truncated syringe, followed by a layer of the rehydrated, sterile substrate, subsequently covered with a sterile mesh secured by a cable tie. Assembled Flowpots were sterilized on a 25 min liquid cycle, stored at 60 °C overnight and sterilized twice on a 45 min solid cycle. Bacterial strains, cultivated as described before, were harvested, 3x washed and pooled in equal ratios. Then, 1.25 ml bacterial pool (OD_{600} 1.0) were added to 500 ml of ½ MS medium for a final bacterial OD_{600} of 0.0025. Flowpots were first flushed with sterile MiliQ water, then inoculated with 50 ml of ½ MS. Eight inoculated Flowpots were placed into each sterile microbox (TP1200, Sac O2) and stored at room temperature overnight. Exactly five sterilized seeds were sown per Flowpot and left to grow under the previously described conditions. At 21 dpi, shoot fresh weight was measured individually for each plant. Roots from a single Flowpot were thoroughly cleaned from soil particles in sterile water using tweezers. Six representative Flowpots were selected for harvesting root and matrix samples. Cleaned roots from each Flowpot were pooled in 2 ml lysing matrix E tubes (MP Biomedicals), snap-frozen and stored at -80 °C until further use. Additionally, <100 mg of soil were taken from each Flowpot, snap-frozen and stored in weighed 2 ml lysing matrix E tube (MP Biomedicals) at -80 °C until further use.

Mono-association experiment of R401 on A. thaliana seedlings in agar plates

This protocol is adapted from (25). In brief, *A. thaliana* seeds were sterilized, germinated, and transferred to ½ MS agar plates without sucrose, as described before. After transfer of seedlings, plants were grown for another 14 days under the same conditions. R401 wild-type and mutants were grown in 50% TSB overnight as described before. Bacterial cells were pelleted by centrifugation, washed 3x in 10 mM $MgCl_2$ and OD_{600} was measured and adjusted to 0.0001. Agar plates were flushed with 15 ml of bacterial suspension for 5 min.

The bacterial suspension were removed, and plants were carefully transferred to new ½ MS agar plates. After 24 h, roots were cut using a sterile scalpel and collected in pre-weighed, sterile 2 ml tubes containing 1 steel bead (3 mm diameter). Tubes were weighed again to assess the root fresh weight. Subsequently, roots were ground in a Precellys 24 TissueLyser (Bertin Technologies) for 2 x 30 s at 6,200 rpm at 15 s intervals. Then, 250 µl of sterile 10 mM $MgCl_2$ were added to each tube and roots were ground again under the same conditions. Each sample was subsequently 5x 1:10 diluted in sterile 10 mM $MgCl_2$. Undiluted samples and each dilution were plated on 50% TSA square plates, dried and left to grow at 25 °C until single colonies appeared. Pictures were taken and single colonies were counted blinded.

Mono-association experiment of R401 on A. thaliana seedlings in Flowpots

Flowpots were assembled, autoclaved, and flushed with sterile MiliQ water, as described before. R401 wild-type and the Δ*pvdl*Δ*phld* double mutant were grown in 50% TSB overnight as described previously. Bacterial cells were pelleted by centrifugation, washed three times in 10 mM $MgCl_2$ and OD_{600} was measured and adjusted. Finally, 600 µl of OD_{600} 1 were inoculated into 300 ml ½ MS medium resulting in a final bacterial concentration of OD_{600} 0.002. Each Flowpot was flushed with 50 ml of ½ MS containing the bacterial inoculum. Per condition, six Flowpots were placed in a sterile microbox (Sac O2) and stored overnight. The next morning, exactly five seeds were inoculated per Flowpot and left to grow under the previously described conditions. After 21 days, roots were carefully cleaned as described above, dried and collected in pre-weighed, sterile 2 ml tubes containing 1 steel bead (3 mm diameter). Tubes were weighed again to assess the root fresh weight. Subsequently, roots were ground in a Precellys 24 TissueLyser (Bertin Technologies) for 2 x 30 s at 6,200 rpm with 15 s intervals. Then, 150 µl of sterile 10 mM $MgCl_2$ were added to each tube and roots were ground again under the same conditions. Each sample was subsequently dilute five times 1:10 in sterile 10 mM $MgCl_2$. Undiluted samples and each dilution were plated on 50% TSA square plates, dried and left to grow at 25 °C until single colonies appeared. Pictures were taken and single colonies were counted blinded.

652

653 *DNA isolation*

654 For DNA extractions, root and soil samples were homogenized in a Precellys 24
655 TissueLyser (Bertin Technologies) for 2 x 30 s at 6,200 rpm with 15 s intervals. DNA was
656 extracted with a modified, high-throughput version of the FastDNA SPIN kit for Soil (MP
657 Biomedicals). In brief, samples were taken up in sodium phosphate buffer (MP
658 Biomedicals) and MT buffer (MP Biomedicals), then homogenized as described before.
659 After centrifugation for 15 min at 13,000 rpm, 150 µl of the supernatant were transferred
660 to a 96-well Acroprep Advance filter plate (with 0.2 µm Supor filter; Pall). Once full, the
661 filter plate was positioned on a PCR plate filled with 50 µl Binding Matrix (MP
662 Biomedicals) per well and centrifuged for 15 min to remove residual soil particles. This
663 and all subsequent centrifugation steps were carried out in a swing out centrifuge at 1,500
664 rpm. The PCR plate was sealed and shaken for 3 min to allow binding of the DNA to the
665 Binding Matrix. The suspension was pipetted onto a second filter plate of the same kind,
666 positioned on a collection plate, and centrifuged for 15 min. The flowthrough was
667 discarded. Then, 200 µl SEWS-M washing buffer (MP Biomedicals) were pipetted into
668 each well of the filter plate and centrifuged for 5 min. This washing step was carried out a
669 second time. The flowthrough was discarded and followed by centrifugation for 5 min to
670 remove residual SEWS-M buffer. Finally, 30 µl nuclease-free water were added to each
671 well and left to incubate at room temperature for 3 min. Subsequent centrifugation for 5
672 min eluted the DNA into a clean PCR plate. The resulting DNA was used for v5v7 *16S*
673 rRNA region amplification without prior adjustment of DNA concentrations.

674

675 *Library preparation for bacterial 16S rRNA gene profiling*

676 The v5v7 variable regions of the bacterial *16S* rRNA gene were amplified in 96-wells
677 plates through PCR using 799F and 1192R primers (PCR I; **Table S4**). PCR I was
678 performed with 0.4 µl DFS-Taq polymerase (BIORON) in 25-µl reactions containing 2.5
679 µl l0x incomplete buffer (BIORON), 0.5 µl l0 mM $MgC1_2$, 2.5 µl 3% BSA, 0.5 µl 10 mM
680 dNTPs, 0.75 µl 10 µM 799F, 0.75 µl 10 µM 1192R, 1 µl of isolated DNA and adjusted to
681 25 µl with nuclease-free water. Samples were placed in a thermocycler set at the following

682 program: 94 °C for 2 min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, then
683 a final extension at 72 °C for 10 min. Remaining primers and dNTPs were digested by
684 Antarctic phosphatase and Exonuclease I. To each 25 µl PCR reaction, 1 µl of Antarctic
685 phosphatase (New-England BioLabs), 1 µl of Exonuclease I (New-England BioLabs) and
686 3 µl of Antarctic phosphatase buffer (New-England BioLabs) were added; the resulting
687 mixture was incubated at 37 °C for 30 min followed by heat inactivation of the enzymes at
688 85 °C for 15 min. The digested PCR I product was centrifuged at 3,000 rpm and 4 °C for
689 10 minutes. Then, 3 µl of the supernatant were used as a template in a second PCR, using
690 a unique combination of uniquely barcoded 799F- and 1192-based primers containing
691 Illumina adaptors for each sample (PCR II; **Table S4**). PCR II was performed with 0.4 µl
692 DFS-Taq polymerase in 25-µl reactions containing 2.5 µl 10x incomplete buffer, 0.5 µl 10
693 mM $MgC1_2$, 2.5 µl 3% BSA, 0.5 µl 10 mM dNTPs, 0.75 µl 10 µM unique forward primer,
694 0.75 µl 10 µM unique reverse primer, 3 µl cleaned PCR I-product and adjusted to 25 µl
695 with nuclease-free water. Samples were placed in a thermocycler set at the following
696 program: 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, then
697 a final extension at 72 °C for 10 min. Subsequently, 5 µl of the PCR product were combined
698 with 1 ml Orange DNA Loading Dye, loaded on a 1% agarose gel containing 0.05% EtBr,
699 and run at 110 mV. The expected PCR-product was an approx. 500 bp band containing the
700 variable v5v7 regions, barcodes, and Illumina adaptors. After verification of successful
701 amplification, samples were purified by AMPure XP (Beckman-Coulter) according to the
702 manufacturer's protocol. The DNA concentrations of each sample were quantified using
703 the Quant-iT dsDNA Assay-Kit (Invitrogen) and pooled per full factorial replicate in
704 equimolar values. All pools were purified twice using AMPure XP and fluorescently
705 quantified using the Quant-iT dsDNA Assay-Kit. All pooled samples were combined into
706 a single pool based on equimolarity and subsequently purified three times using AMPure
707 XP and fluorescently quantified using the Qubit dsDNA HS Assay Kit (Invitrogen). The
708 final concentration was set to 10 ng/µl. Paired-end Illumina sequencing was performed in-
709 house using the MiSeq sequencer and custom sequencing primers (**Table S4**).

710

Analysis of 16S profiling data

ASV table generation

Amplicon sequencing reads from *A. thaliana* roots and Flowpot soil were quality filtered and demultiplexed according to their barcode sequence using QIIME (26) and unique amplicon sequencing variants (ASVs) were inferred from error-corrected reads, followed by chimera filtering. ASVs were mapped to the reference 16S rRNA sequences (downloaded from "www.at-sphere.com") to generate an ASV count table. All steps were carried out using the Rbec R package (27). Analysis was performed on samples with a sequencing depth of at least 500 high-quality reads.

Alpha- and beta-diversity

Analyses and visualization were performed in the R statistical environment (Version 4.1.2). Alpha and beta diversity were calculated on non-rarefied ASV count tables (28). Alpha-diversity (Shannon index) was calculated with the "plot_richness" function in phyloseq package (29).

Beta-diversity (Bray-Curtis dissimilarities) was calculated using the "ordinate" function in phyloseq package and used for unconstrained ordination by Principal Coordinate Analysis (PCoA). Statistical significances were assessed using permutational multi-variate analysis of variance (PERMANOVA) using the adonis function in the vegan package (30).

Data analysis and statistics

All statistical analyses were conducted in R 4.1.2. Data visualisation was conducted using the ggplot2 package (as part of the Tidyverse) or the ggpubr package. Data normality was tested using the Shapiro-Wilk test. For normally distributed data, ANOVA and Tukey's HSD with correction for multiple comparisons were used. As nonparametric tests, Kruskal-Wallis followed by Dunn's post-hoc test and Benjamini-Hochberg (BH) adjustment for multiple comparisons were used. The respective statistical tests are indicated in each figure description. Significance was indicated by asterisks (∗, ∗∗, and ∗∗∗, indicate p.adj ≤ 0.05, 0.01, and 0.001, respectively) or by significance group ($p \leq 0.05$). No statistical methods

were used to pre-determine sample sizes. Phylogenetic trees were computed using Clustal Omega and subsequently visualised using iTol. Metabolomics and *16S* rRNA gene profiling data were analysed and visualized as described above. Halo size quantification in mBA experiments were performed blinded using the Fiji package of ImageJ. Colony counts of R401 were performed blinded. Figures were assembled in Adobe Illustrator.

References

1. Y. Bai, *et al.*, Functional overlap of the Arabidopsis leaf and root microbiota. *Nature 2015 528:7582* **528**, 364–369 (2015).
2. A. Boucher, *et al.*, Transposon mutagenesis of Pseudomonas solanacearum: isolation of Tn5induced avirulent mutants. 131--2449 (1985).
3. D. Aldon, B. Brito, C. Boucher, S. Genin, A bacterial sensor of plant cell contact controls the transcriptional induction of Ralstonia solanacearum pathogenicity genes. *EMBO J* **19**, 2304 (2000).
4. M. Wang, *et al.*, Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat Biotechnol* **34**, 828–837 (2016).
5. A. T. Aron, *et al.*, Reproducible molecular networking of untargeted mass spectrometry data using GNPS. *Nature Protocols 2020 15:6* **15**, 1954–1991 (2020).
6. M. J. Chaisson, G. Tesler, Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): Application and theory. *BMC Bioinformatics* **13**, 1–18 (2012).
7. M. Kolmogorov, J. Yuan, Y. Lin, P. A. Pevzner, Assembly of long, error-prone reads using repeat graphs. *Nat Biotechnol* **37**, 540–546 (2019).
8. T. Seemann, Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069 (2014).
9. R. R. Wick, M. B. Schultz, J. Zobel, K. E. Holt, Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* **31**, 3350–3352 (2015).
10. M. Seppey, M. Manni, E. M. Zdobnov, BUSCO: Assessing genome assembly and annotation completeness. *Methods in Molecular Biology* **1962**, 227–245 (2019).

11. R. Challis, E. Richards, J. Rajan, G. Cochrane, M. Blaxter, BlobToolKit – Interactive Quality Assessment of Genome Assemblies. *G3 Genes|Genomes|Genetics* **10**, 1361–1374 (2020).
12. K. Blin, *et al.*, antiSMASH 6.0: improving cluster detection and comparison capabilities. *Nucleic Acids Res* **49**, W29–W35 (2021).
13. D. G. Gibson, *et al.*, Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* **6**, 343–345 (2009).
14. B. H. Kvitko, A. Collmer, Construction of Pseudomonas syringae pv. tomato DC3000 mutant and polymutant strains. *Methods Mol Biol* **712**, 109–128 (2011).
15. D. S. Merrell, D. L. Hava, A. Camilli, Identification of novel factors involved in colonization and acid tolerance of Vibrio cholerae. *Mol Microbiol* **43**, 1471–1491 (2002).
16. C. Leys, C. Ley, O. Klein, P. Bernard, L. Licata, Detecting outliers: Do not use standard deviation around the mean, use absolute deviation around the median. *J Exp Soc Psychol* **49**, 764–766 (2013).
17. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, Basic local alignment search tool. *J Mol Biol* **215**, 403–410 (1990).
18. E. Martínez-García, *et al.*, SEVA 3.0: an update of the Standard European Vector Architecture for enabling portability of genetic constructs among diverse bacterial hosts. *Nucleic Acids Res* **48**, D1164–D1170 (2020).
19. N. K. Arora, M. Verma, Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech* **7** (2017).
20. B. Schwyn, J. B. Neilands, Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56 (1987).
21. E. Baudoin, E. Benizri, A. Guckert, Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biol Biochem* **35**, 1183–1192 (2003).
22. T. Thiergart, *et al.*, Root microbiota assembly and adaptive differentiation among European Arabidopsis populations. *Nat Ecol Evol* **4**, 122–131 (2020).

23. K. Wippel, *et al.*, Host preference and invasiveness of commensal bacteria in the Lotus and Arabidopsis root microbiota. *Nature Microbiology 2021 6:9* **6**, 1150–1162 (2021).
24. J. M. Kremer, *et al.*, Peat-based gnotobiotic plant growth systems for Arabidopsis microbiome research. *Nature Protocols 2021 16:5* **16**, 2450–2470 (2021).
25. K. W. Ma, *et al.*, Coordination of microbe–host homeostasis by crosstalk with plant innate immunity. *Nature Plants 2021 7:6* **7**, 814–825 (2021).
26. J. G. Caporaso, *et al.*, QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336 (2010).
27. P. Zhang, S. Spaepen, Y. Bai, S. Hacquard, R. Garrido-Oter, Rbec: a tool for analysis of amplicon sequencing data from synthetic microbial communities. *ISME Communications 2021 1:1* **1**, 1–3 (2021).
28. P. J. McMurdie, S. Holmes, Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS Comput Biol* **10**, e1003531 (2014).
29. P. J. McMurdie, S. Holmes, phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* **8**, e61217 (2013).
30. P. Dixon, VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science* **14**, 927–930 (2003).