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Chapter 6. Differential effects of plant hormones on growth of *Streptomycetaceae* and other plant-associated bacteria

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Abstract

Jasmonic acid (JA) is a plant hormone that affects the growth and natural product biosynthesis in Streptomycetes. Conjugation of JA by *Streptomyces roseifaciens* to jasmonoyl glutamine (JA-Gln) led to increased JA tolerance. However, whether other *Streptomycetaceae* genera use this mechanism to neutralize the toxicity of plant hormones is not known. Here, we show differential sensitivity between *Streptomyces* and *Streptacidiphilus* species towards the plant hormones JA, indole acetic acid (IAA) and salicylic acid (SA). In response to JA exposure, both *Streptacidiphilus* and *Streptomyces* species conjugated JA to various amino acids, which resulted in reduced JA toxicity. However, addition of 0.5 mM JA inhibited the growth of most *Streptacidiphilus* species, while growth of streptomycetes was not affected. This selective pressure of phytohormones on growth was also observed for microbial communities isolated from bulk soil, rhizosphere and plant *Arabidopsis* roots. An overall drop in colony forming units was observed when phytohormones were added to the medium. In contrast, colonies of *Paraburkholderia* and *Burkholderia* species were prevalent. These results suggest that phytohomones may function as bacterial gatekeepers, favoring plant entry by specific root-associated bacteria with higher phytohormone tolerance.

Introduction

Plants host a plethora of microorganisms which have important roles for plant growth and health (Mendes, Garbeva, and Raaijmakers 2013). Endophytes are beneficial microbes living inside the plant, thereby being part of the endophytic community. These microbes are either vertically transferred to the plant seedling or recruited from the rhizosphere, the narrow region of soil that is directly influenced by root secretions and its associated soil microorganisms. The plants rhizosphere microbiome is largely dependent on soil type and plant genotype (Mendes, Garbeva, and Raaijmakers 2013; Cordovez et al. 2019). As such, rhizodeposits are known to attract and support growth of beneficial microbes present in the soil, whereas they repel and inhibit growth of others (Yu et al. 2019; Mendes, Garbeva, and Raaijmakers 2013). Plant hormones indirectly play a role in shaping the plants microbiome by influencing the composition of root exudates and (in)activation of plant defense systems. Indeed, alterations in JA- and SA signaling was shown to affect the root microbiome of *Arabidopsis thaliana* (Carvalhais, Schenk, and Dennis 2017; Lebeis et al. 2015).

Actinobacteria constitute a significant part of the root microbiome across the plant kingdom, which is largely determined by an increased relative abundance of the family of *Streptomycetaceae* (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012; Chapter 3). Previously, we showed that the relative abundance of the actinobacteria inhabiting the endophytic compartment of wild *A. thaliana* is over 20%, mainly driven by enrichment of Operational Taxonomic Units (OTUs) classified as *Streptomycetaceae* (Chapter 3). The family of *Streptomycetaceae* is comprised of three genera: *Streptomyces*, *Kitasatospora*, and *Streptacidiphilus* (Labeda et al. 2017). Their multicellular hyphae grow by a combination of tip extension and branching, thereby forming an intricate mycelial network. Their developmental lifecycle is completed by the production of aerial hyphae that eventually form chains of uni-genomic spores (Barka et al. 2016; Claessen et al. 2014). *Streptomycetaceae* can inhabit a wide range of ecological niches (Kämpfer et al. 2014), whereas *Streptacidiphilus* bacteria typically grow at lower pH ranges than *Streptomyces* and *Kitasatospora*. Hence, *Streptacidiphilus* strains are isolated from acidic soils and litter, whereas

Streptomyces species are known to thrive in soil, sea- and fresh water systems, or live as symbionts of higher eukaryotes (Kim et al. 2003; Chapter 2; Seipke, Kaltenpoth, and Hutchings 2012). Members of the genus *Kitasatospora* differ from streptomycetes by the composition of their cell wall. *Kitasatosporae* contain both LL- and meso-diaminopimelic acid in the amino acid chains of the peptidoglycan, whereas *Streptomyces* species contain exclusively LL-diaminopimelic acid (Takahashi 2017). Genetic and phylogenetic approaches have confirmed that *Kitasatospora* and *Streptacidiphilus* are distinct from *Streptomyces*, and cluster outside of the phylogenetic radiation of the genus *Streptomyces* (Labeda et al. 2017; Girard et al. 2013).

Many *Streptomyces* species are plant-associated, thereby fulfilling a role as (endo)symbiont, saprophyte or pathogen (Viaene et al. 2016). Plant-associated *Streptomyces* are predominantly recognized for their beneficial effect on plant health due to their ability to produce a wide variety of secondary metabolites, including siderophores for iron acquisition as well as antibacterial and antifungal compounds which help them to protect their host, which in turn may contribute to their success in inhabiting the rhizosphere. Some plant-associated *Streptomyces* produce plant hormones, or even grow on these hormones as sole carbon source (Lebeis et al. 2015; Lin and Xu 2013; Viaene et al. 2016). As alternatives to chemical pesticides are becoming increasingly desirable, the potential of *Streptomyces* to protect plants in a sustainable way is more and more recognized (Newitt et al. 2019).

Our previous work showed that plant hormones jasmonic acid (JA), indole acetic acid (IAA) and salicylic acid (SA) alter antibiotic production of plant-associated *Streptomyces* (Chapter 3). These results support the *cry for help* hypothesis, which states that plants under siege activate antimicrobial production in their microbiome for protection. Additional work showed an effect of JA on development of *Streptomyces coelicolor*, resulting in accelerated sporulation. Despite these phenotypic and physiological effects of JA, it also imposes a toxic effect on *Streptomyces*. To neutralize this toxic effect, *Streptomyces roseifaciens* conjugates JA to the amino acid glutamine, resulting in jasmonoyl-glutamine (JA-Gln). If these responses to JA and other plant hormones are typical for streptomycetes is yet unknown.

Here, we show conjugation of JA to various amino acids by both *Streptomyces* and *Streptacidiphilus* species. In general, *Streptacidiphilus* strains grew poorly on JA compared to *Streptomyces* bacteria, which was also the case for SA and IAA. We further show that plant hormones can add selective pressure on microbial communities from soil, rhizosphere and endosphere. We observed an overall reduction in colony forming units on plates with increasing concentrations of phytohormones and a concomitant higher frequency of *(Para)Burkholderia* species. These results suggest that plant hormones can aid in the recruitment and assembly of the plants' microbiome.

Materials and Methods

Strains and growth conditions

Streptacidiphilus

Bacteria were isolated from decaying wood on water-yeast agar pH5 (Scheublin et al, 2020). *Streptacidiphilus* strains were grown on either ½ PDA (2 mg/L potato extract, 10 g/L dextrose, 20 g/L Iberian agar) supplemented with 25 mM MES, pH 5.5, or on ISP4 supplemented with 25 mM MES, pH 5.5 to obtains spore stocks. To test plant hormone tolerance, spots of approx. 2 µL were added on ½ PDA by using a replicator. The agar plates were supplemented with (±)-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7), 3-indoleacetic acid (Sigma-Aldrich, CAS No.: 87-51-4) or salicylic acid (Alfa Aesar, CAS No.: 69-72-7), for which 10% w/v solutions were prepared with EtOH as solvent.

Streptomyces

Streptomyces coelicolor A3(2) M145 was obtained from the John Innes Centre strain collection in Norwich, UK. Isolation of soil and plant associated *Streptomyces* was described previously (Zhu et al. 2014; Chapter 3). *Streptomyces* were grown on SFM for 5 days at 30 °C to obtain spore stocks. To test plant hormone tolerance, spots of approx. 2 µL of ~10^7 spores/mL were added by using a replicator to minimal medium (Kieser T. 2000) with mannitol (0.5%) and glycerol (1%) as carbon sources and 25mM TES buffer. The agar plates were supplemented with (±)-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7), 3-indoleacetic acid (Sigma-Aldrich, CAS No.: 87-51-4) or salicylic acid (Alfa Aesar, CAS No.: 69-72-7), for which 10% w/v solutions were prepared with EtOH as solvent.

Arabidopsis thaliana

A sterile *A. thaliana* ecotype Columbia (Col-0) was used for the recruitment of endophytic actinobacteria under lab conditions. Plants were grown on a mixture of 9:1 substrate soil and sand (Holland Potgrond) at 21 °C, a 16h

photoperiod, and 70% relative humidity. After 2 weeks of growth, the plants were harvested.

Extraction of secondary metabolites and LC-MS

Bacterial culturing and extraction

A total of 16 *Streptomyces* strains were grown in 25 mL of liquid TSBS medium (tryptic soy broth 30 g/L, sucrose 100 g/L, pH 7), either with or without the addition of 0.5 mM jasmonic acid (1 mL/L). The cultures were incubated at 30 °C and shaken at 200 rpm. On the other hand, 13 *Streptacidiphilus* strains were grown in 25 mL of liquid TSB supplemented with 25 mM MES (tryptic soy broth 30 g/L, MES 1.95 g/L, pH 5), either with or without the addition of 0.5 mM JA (1 mL/L). The cultures were incubated at 20 °C and shaken at 180 rpm. Bacteria growing without JA were supplemented with 0.1% EtOH, which is the solvent used for JA. After 3 – 7 days, 5% w/v Diaion® HP20 (Resindion) was added to the cultures and shaken for three hours. HP20 and the mycelia were filtered off the liquid media, washed with distilled water, then extracted with MeOH three times by overnight soaking. The MeOH extracts were then dried under reduced pressure, and re-dissolved in 90% MeOH in H_2O to a final concentration of 3 mg/mL for LC-MS analysis. The liquid media used for culturing, with either JA or EtOH, were similarly prepared and extracted to serve as negative controls.

LC-MS measurement

LC-MS spectra were obtained using a Waters Acquity UPLC system coupled to a Thermo Instruments MS system (LTQ Orbitrap XL). The UPLC system was run using SunFire C_{18} column (3.5 µm, 100 Å, 4.6 × 150 mm). Solvent A was 0.1% formic acid, 95% H_2O and 5% acetonitrile (ACN). Solvent B was 0.1% formic acid and 100% ACN. The gradient used was 2% B for 1 min, 2-85% for 15 min, 85-100% for 3 min, and 100% for 3 min. The flow rate used was 0.9 mL/min. A fixed injection volume of 5 µL was used for all the analyzed extracts. The MS conditions used were as followed: spray voltage 3.5 kV, capillary voltage 5 V, capillary temperature 300 °C, auxiliary gas flow rate 10 arbitrary units, and sheath gas flow rate 50 arbitrary units. The mass analyzer was calibrated using Agilent low concentration tuning mix before each run. The MS method used

for molecular networking comprised of full scan MS in the orbitrap in the positive mode at a mass range 100-2000 *m/z* and FT resolution of 30000, followed by data dependent MS^2 in the ion trap for the three most intense ions using collision induced dissociation (CID) with normalized collision energy of 35 and isolation width of 1.5 *m/z*. Dynamic exclusion was enabled after three counts with an exclusion duration set to 10 s. A run was also performed with data dependent MS^2 being scanned in the orbitrap at an FT resolution of 7500, to deduce the molecular formulae of fragment ions of interest.

Molecular networking

Raw MS/MS data were centroided and converted to 32-bit uncompressed mzXML files using MSConvert (ProteoWizard) (Chambers et al. 2012). The data were then uploaded to the GNPS platform(https://gnps.ucsd.edu) for molecular networking (Wang et al. 2016). The parameters used for molecular networking were a parent and fragment ions tolerance of 0.5 Da, a minimum cosine score of 0.5, minimum matched peaks of 4, maximum node size of 200, and MSCluster enabled with a minimum cluster size of 3 spectra. The spectra were also searched against GNPS spectral libraries using default settings. Group and Attributes files were generated according to the GNPS documentation, and the generated molecular networks were visualized in Cytoscape (Shannon et al. 2003). The molecular networks on GNPS can be accessed at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=1a8d97d086 1944a8990454f6-a04aa581.

Synthesis of jasmonic acid and jasmonic acid amino acid conjugates

Jasmonic acid and JA amino acid conjugates were prepared according to Jikamaru et al., 2004.

Racemic jasmonic acid

To a solution of racemic methyl jasmonate (JA-Me; 4.85 mL, 22.3 mmol) in methanol (MeOH; 50 mL), a 5 M KOH aqueous solution (7.5 mL) was added while stirring at room temperature for 5 h. The reaction mixture was neutralized with 6 M aqueous HCl and concentrated *in vacuo*. The residue was

dissolved in H_2O (50 mL), and the solution adjusted to pH 2 – 3 with 6 M aqueous HCl, before being extracted with ethyl acetate (50 mL, 3 times). The combined organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified with a column of silica gel using a mixture of n-hexane:ethyl acetate:acetic acid (14:6:1, v/v/v) as an eluent, to give racemic-JA (3.8 g, 18 mmol, 75% yield). NMR was in accordance with the reference.

Jasmonic acid-N-hydroxysuccinimide ester

To a mixture of racemic jasmonic acid (1 g, 4.8 mmol) in ACN (10 mL) and *N*-hydroxysuccinimide (1.5 g, 13 mmol) in *N,N*-dimethylformamide (DMF; 7.5 mL), dicyclohexylcarbodiimide (DCC; 1.25 g, 6.1 mmol) in ACN (5 mL) was added while stirring at room temperature for 48 h. Water (20 mL) was added to decompose the excess DCC, and the reaction mixture was filtered to remove the dicyclohexylurea. The filtrate was concentrated *in vacuo* and purified with a column of silica gel using a mixture of n-hexane:ethyl acetate (1:3, v/v) as an eluent, to give the jasmonic acid–*N*-hydroxysuccinimide ester (1.2 g, 3.9 mmol, 81% yield). NMR was in accordance with the reference.

General procedure for preparing jasmonic acid-L-amino acid conjugates

Jasmonic acid-*N*-hydroxysuccinimide ester (200 mg, 0.65 mmol) in ACN (10 mL) was mixed with a solution of 1 mmol amino acid (L-valine, L-isoleucine, L-tyrosine, L-phenylalanine and L-glutamine) in H_2O (10 mL). To this mixture, triethylamine (1 mL, 8.5 mmol) was added while stirring at room temperature for 16 h. The resulting reaction mixture was concentrated *in vacuo*. The concentrate was dissolved in 0.1 M aqueous HCl (50 mL) and extracted with ethyl acetate (50 mL, three times). The combined ethyl acetate layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by a column of silica, using a mixture of ethyl acetate:acetic acid (99:1, v/v) as an eluent, to give the racemic jasmonic acid-L-amino acid conjugate. All NMRs were in accordance with previously reported data.

Filter disk assays

Tripartite Petri dishes were filled with 6 mL MM with 0.5% mannitol and 1% glycerol (*Streptomyces*) and ½ PDA (*Streptacidiphilus*) per compartment. Agar

was confluently streaked by applying 2 µL spore stock of *Streptomyces* and 10 µL of *Streptacidiphilus* stock. Next, sterilized filters were applied, on which 10 µL of jasmonic acid (10%), apramycin (0.5 mg/mL) or DMSO was added. Pictures were taken after two days of growth.

Isolation of bacteria in the presence of plant hormones

To isolate *Streptomycetaceae* from soil, rhizosphere, or endosphere, 100 µL of either a potting soil:sand mixture (approx. 10 mg/mL), rhizosphere suspension (approx. 1 mg/mL) or suspension of surface sterilized *A. thaliana* Col-0 roots, were plated on minimal medium (Kieser T. 2000) with mannitol (0.5%) and glycerol (1%) as carbon sources, nystatin (5 µg/mL), nalidixic acid (5µg/mL) and 25mM TES buffer. The agar plates were supplemented with (±)-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7), 3-indoleacetic acid (Sigma-Aldrich, CAS No.: 87-51-4) or salicylic acid (Alfa Aesar, CAS No.: 69-72-7), for which 10% w/v solutions were prepared with EtOH as solvent. The plates were typically incubated for 7 days at room temperature. Isolates were regrown on MM and stored in 20% glycerol at -20 °C.

PCR

The 16S gene fragments were amplified by PCR from biomass cooked in MQ for 15 min using the primers FW:CAGGCCTAACACATGCAAGTC annealing to position 63 of the gene and RV:ACGGGCGGTGTGTACAAG to position 1389. Sequencing was done from position 515 with primer FW:GTGYCAGCMGCCGCGGTAA at BaseClear in Leiden, the Netherlands. Sequences were compared to the NCBI database.

Results

Streptacidiphilus has a lower tolerance to plant hormones than *Streptomyces*

We previously showed that JA affected the growth of *S. coelicolor* and *S. roseifaciens.* Here, we tested a collection of 32 *Streptomycetaceae*, including 22 *Streptomyces* strains and 11 *Streptacidiphilus* strains (Scheublin et al. 2020) for their sensitivity to phytohormones. All strains were grown in the presence of 0.5 mM JA, 0.6 mM IAA or 0.7 mM SA. In accordance with previous experiments, 19 out of the 22 tested *Streptomyces* isolates grew well on all types of plant hormones (Figure 30A) In contrast, most of the tested *Streptacidiphilus* strains grew poorly when the plant hormones were added to the medium (Figure 30B). Only five *Streptacidiphilus* strains grew on either JA or IAA, and only one strain grew on SA. *Streptacidiphilus* strains that did grow on either one or more of the plant hormones showed reduced biomass as compared to the control plates without phytohormones. To get more detailed insights into the plant hormone tolerance amongst the *Streptacidiphilus* isolates, we estimated the MIC of JA, IAA and SA for a strain that was susceptible to all plant hormones (Sc1) and one that was relatively resistant (Sc2) (Figure 30B). Both Sc1 and Sc2 showed then some growth on 0.5 mM JA, while Sc2 showed limited growth up to 1 mM (0.02%) (Figure S21). This suggests that JA tolerance amongst our *Streptacidiphilus* isolates revolves around 0.5 mM. Moreover, Sc2 could grow on four times as much SA than Sc1, indicating that SA tolerance varies amongst *Streptacidiphilus* bacteria (Figure S21). On the other hand, IAA tolerance was similar, with both strains growing well on 0.3 mM (0.05%) and Sc2 growing poorly up to 0.6 mM (0.1%). An additive toxicity effect of the plant hormones was observed when they were administered at the same time (Figure S21).

As for *Streptomyces*, the tested strains grew on media with up to 10 times higher concentrations of JA (5 mM) or SA (7 mM) compared to *Streptacidiphilus*, while IAA was relatively more toxic even at the initially tested concentration of 0.6 mM, with significant growth reduction in comparison to the control (Table S4). In a control experiment, *Streptacidiphilus* and *Streptomyces* showed comparable tolerance towards

apramycin and DMSO, suggesting that the low tolerance to plant hormones was not due to a general sensitivity to toxic compounds by *Streptacidiphilus* (Figure S22). Together these results show differential sensitivity towards plant hormones between *Streptomyces* and *Streptacidiphilus*.

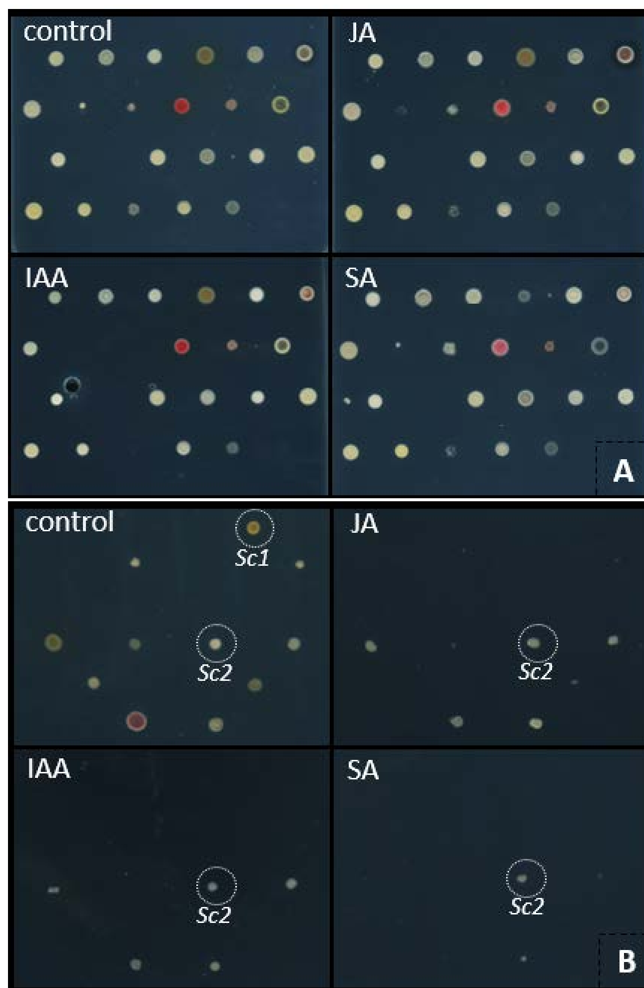

Figure 30: **Phytohormones are generally more toxic to *Streptacidiphilus* than to *Streptomyces*.** Strains were grown for four days with or without plant hormones, using MM for Streptomyces and ½ PDA for *Streptacidiphilus*. *Streptomyces* strains generally showed growth on 0.5 mM JA, 0.6 mM IAA or 0.7 mM SA (A), while most *Streptacidiphilus* species did not grow at these concentrations (B). *Streptacidiphilus* strain Sc1 did not grow on plant hormones at all, whereas strain Sc2 did.

JA is conjugated to various amino acids by *Streptomyces* and *Streptacidiphilus*

Previously, JA conjugation to JA-Gln by *Streptomyces roseifaciens* was observed, which in contrast to JA did not exert selective pressure on the bacterium (Chapter 5). To get insight into the distribution of this trait amongst *Streptomycetaceae*, and whether this could be linked to the differences in JA sensitivity, a selection of 16 *Streptomyces* and 13 *Streptacidiphilus* strains were grown in liquid culture media supplemented with 0.5 mM JA. The *Streptomyces* strains selected comprised seven lab strains (two *Streptomyces coelicolor* variants, *Streptomyces venezuelae*, *Streptomyces scabies*, *Streptomyces griseus*, *Streptomyces lividans*, and *Streptomyces flavogriseus*), six plant-associated isolates (*Streptomyces* spp. Atmos39, Atmos31, AC109, AC107, Endo68, and Endo57), and three soil isolates (*S. roseifaciens*, *S.* spp. C1 and QL37). Only three *Streptacidiphilus* isolates (*Streptacidiphilus spp.* P03-D6a, P15-A2a and P18-A5a) produced appreciable biomass when grown on JA whereas all *Streptomyces* isolates did, confirming the relatively low JA tolerance of *Streptacidiphilus* compared to *Streptomyces*. The bacterial cultures were then extracted and analyzed using LC-MS/MS.

Since JA-Gln was previously purified and identified from *S. roseifaciens* (Chapter 5), molecular networking was chosen for finding JA-Gln or other structurally related metabolites in the bacterial extracts. This is because molecular networking clusters the metabolites, detected by MS analysis, based on similarities in their fragmentation spectra. Accordingly, the obtained LC-MS/MS data of the bacterial extracts were uploaded to the GNPS platform for generation of molecular networks (Wang et al. 2016). Cytoscape was then used to visualize the obtained network which was made up of 1881 nodes (Figure 31), each node represented a cluster of MS^2 fragmentation spectra for a certain precursor ion. The nodes were connected to each other by an edge if their MS^2 spectra had a minimum of 4 matched peaks, and a minimum cosine score of 0.5. Based on this 1382 nodes were grouped into 210 spectral families.

Of the total observed nodes, 129 were attributed to ions detected only in JA-treated cultures, eight of which were additionally detected in the control growth media with JA and no bacteria (Figure 31). The node associated with

JA-Gln could be observed in the network. Looking at its MS^2 spectrum, it was possible to identify the characteristic fragments **a** – **c**, which are consistent with the loss of a Gln moiety, and further fragmentation of the jasmonoyl moiety (Figure S23). It was also possible to identify the fragment **d** due to the $[M+H]^+$ ion of glutamine. With this information in hand, the nodes exclusively detected in JA-treated cultures were checked for a similar fragmentation pattern. The JA-Gln node formed a network with two other nodes, whose precursor masses were 354.186 and 353.207, and they were both exclusively detected in JA-treated cultures (Figure S23). While the node with a precursor mass of 354.186 turned out to be an artifact, the one with a precursor mass of 353.207 did show an MS^2 spectrum consistent with a JA amino acid conjugate. The mass difference between the 353.207 node and that of JA-Gln indicated a molecule with an extra methyl group. MS^2 spectrum of the 353.207 node still retained the same fragments **a** – **c** due to the jasmonoyl moiety. There was also a fragment due to a loss of HCOOH group as in JA-Gln, but there was no fragment due to a loss in NH_3. Thus, the observed node was annotated as a JA conjugate of *N*-methyl-glutamine (JA-*N*-MeGln).

Another spectral family in the molecular network consisted of four nodes all exclusively detected in JA-treated bacterial cultures (Figure 31). One of the nodes was chosen to investigate the structural feature of the observed group. The selected node had a precursor mass of 374.199, which corresponds to an $[M+H]^+$ ion for a molecule whose molecular formula is $C_{21}H_{27}NO_5$. The MS^2 spectrum of this ion showed that fragments **a** – **c** due to the jasmonoyl moiety were 16 Da higher (Figure S24), which corresponds to an additional oxygen based on the accurate mass measured in the orbitrap. Accordingly, the observed ion was deduced to be an amino acid conjugate of hydroxy-jasmonic acid (OHJA). The amino acid was then deduced to be phenyl alanine, based on the molecular formula of the precursor mass together with the observed fragment **d** due to the amino acid. OHJA and its amino acid conjugates have been previously reported in plants as one of the inactivation pathways for the hormone, and the hydroxylation position was found to be at C-12 (Smirnova et al. 2017; Caarls et al. 2017). A similar position might be assigned to the observed molecule, especially with the observation of the intense fragment ion **e** which is likely due to the loss of the hydroxylated side chain comprising C-9 to C-12 in the jasmonoyl moiety (Figure S24). Further scrutiny of the

molecular network revealed that all the nodes connected to OHJA-Phe had a fragmentation pattern and molecular formula consistent with amino acid conjugates of OHJA. The three additional nodes were thus annotated as OHJA conjugates of valine (Val), tyrosine (Tyr), and Leucine/isoleucine (Leu/Ile) (Figure 31). The network also showed nodes which could be due to conjugates of the same amino acids to JA rather than OHJA, but their intensity was very low, and consequently we failed to obtain enough fragments to cluster closely with JA-Gln or with each other.

Of the strains tested, *S. roseifaciens* showed the highest level of JA-Gln, with a peak that was more intense than any other JA or OHJA conjugate. In addition to *S. roseifaciens*, JA-Gln was also detected in *S. scabies* and *S. lividans* and in the *Streptacidiphilus* sp. P03-D6a and P18-A5a. On the other hand, JA-*N*-MeGln was mainly detected in *S. roseifaciens* and as a minor peak in the plant-associated strain *S.* sp. Atmos39. Preliminary data suggest that *S.* sp. Atmos39 also generates JA-Gln, but the peak intensity was too low to be validated by MS^2 fragmentation.

Amino acid conjugation reduces toxicity of JA

To examine the effect of JA conjugates on *Streptacidiphilus*, our collection was exposed to JA-Gln and JA-Phe. Droplets of 2 µl of *Streptacidiphilus* stocks (10^5 CFU/mL) were spotted onto media containing 0.5 mM of each JA amino acid conjugate (Figure 32). Both amino acid conjugates were less toxic to all the tested *Streptacidiphilus* bacteria compared to JA, showing that conjugation of JA lowers the toxicity towards *Streptacidiphilus*, like was previously shown for *S. roseifaciens*. The MIC values of JA, JA-Phe and JA-Gln were estimated for a *Streptacidiphilus* isolate which was tolerant to 0.5 mM JA (Figure S25A) and another which was unable to grow under the same condition (Figure S25B). Both strains were able to grow on JA-Gln concentrations of up to 3 mM, which was the highest concentration tested. Compared to JA, this is an increase in tolerance of at least six-fold for the JA-tolerant strain. JA-Phe neutralized JA as well, but to a lesser extent, increasing tolerance up to three times (Figure S25).

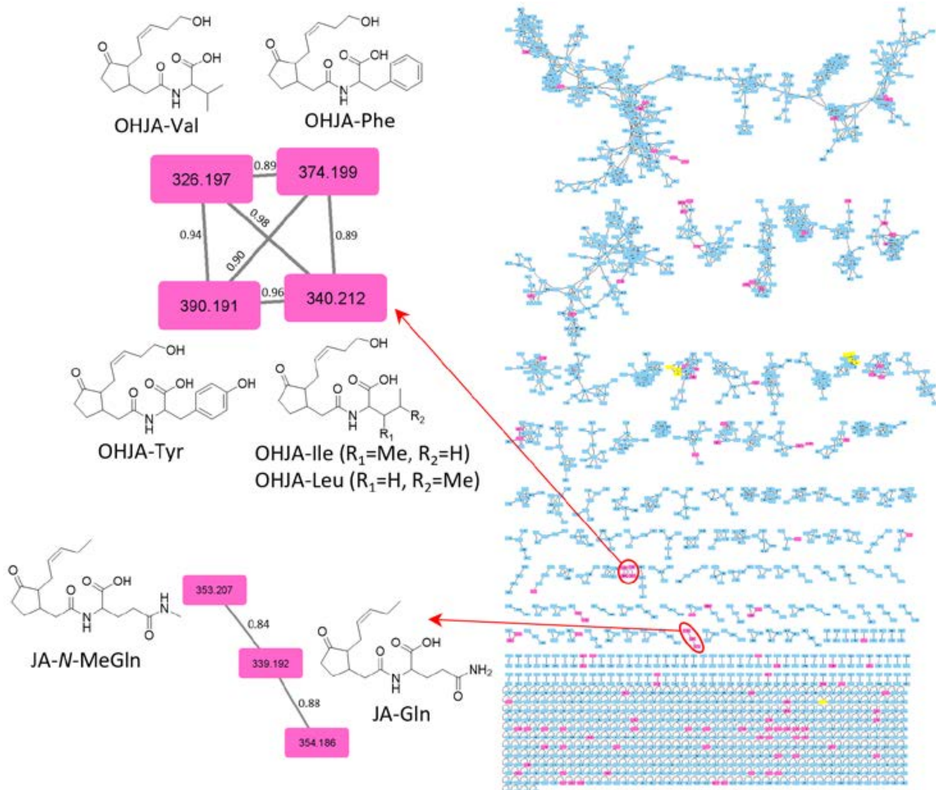

Figure 31: **Molecular network of extracts of Actinobacteria grown on medium with the plant hormone jasmonic acid.** Pink nodes represent ions detected only in jasmonic acid treated actinobacterial cultures, while yellow nodes represent the ions detected in jasmonic acid treated cultures together with blank media. Two networks are enlarged which include amino acid conjugates of both jasmonic acid (JA) and hydroxyjasmonic acid (OHJA). The nodes are labeled by their precursor masses, and the edges by their cosine scores.

Selective effects of JA and other plant hormones on microbial communities from soil, rhizosphere and roots

To investigate whether plant hormones select for *Streptomycetaceae* species among soil and root-associated microbiomes, bulk soil, rhizosphere soil and surface-sterilized *Arabidopsis* roots were plated on MM with a mix of IAA, JA and SA at concentrations of 0.003% (0.17, 0.14 and 0.22 mM, respectively)

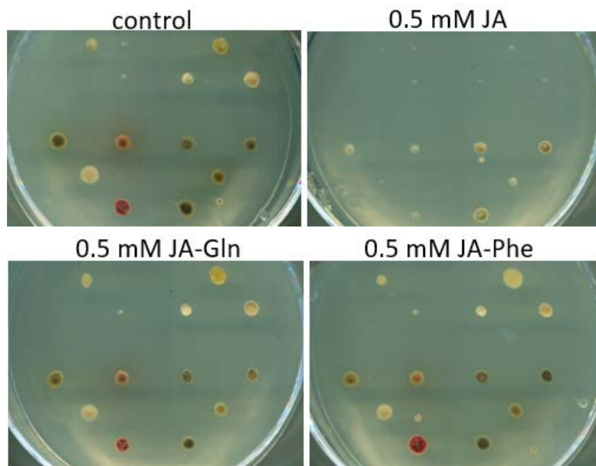

Figure 32: **JA-Gln and JA-Phe are not toxic to *Streptacidiphilus*.** Spots of Streptacidiphilus on ½ PDA and ½ PDA with 0.5 mM JA, 0.5 mM JA-Gln and 0.5 mM JA-Phe after 5 days of growth. 0.5 mM of JA-Gln or JA-Phe is not abolishing growth, whereas JA is.

and 0.03% (1.7, 1.4, and 2.2 mM, respectively). MM agar plates were supplemented with 1% glycerol and 0.5% mannitol as the carbon sources. Nystatin and nalidixic acid (both at 5 µg/mL concentrations) were added to inhibit the growth of fungi and Gram-negative bacteria, respectively. After 14 days of growth at room temperature, approximately 10 times lower CFU counts were obtained on plates supplemented with 0.03% of the plant hormone cocktail (Figure 33). *Streptomycetaceae* colonies were mostly found on control plates or plates with 0.003% of the plant hormone cocktail but were barely detected on plates supplemented with 0.03% of each plant hormone. Instead, the bacteria that were found on those plates had a slimy and creamy colored appearance. 16S rDNA sequencing of those colonies identified particularly *Burkholderia* and *Paraburkholderia* species. Interestingly, these bacteria were also isolated from plates that were inoculated with a surface-sterilized root sample, which suggests that these plant hormone-tolerant species live inside the root system, i.e. endophytes. A follow-up experiment in which the plant hormones were added separately to the plates showed that IAA exerted most of the selective pressure (Figure S26).

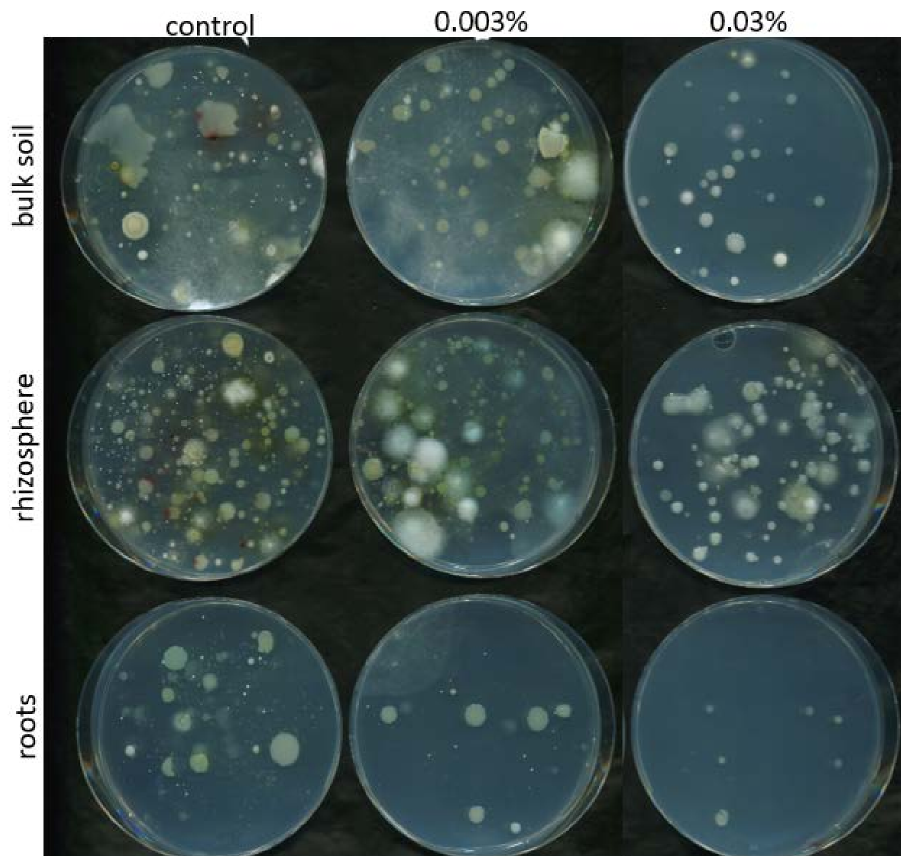

Figure 33: **Increasing the amount of IAA, JA and SA in the agar results in a colony forming unit drop.** CFU's appearing by plating a suspension of sterilized roots are less diverse compared to microbes isolated from the bulk soil or rhizosphere wash. Taking the same suspensions, microbe diversity and CFUs decline when adding 0.003% or 0.03% of SA, IAA and JA (0.003% and 0.03%) compared to the control (control).

Discussion

Plant hormones can influence the plants' microbiome by altering the composition of root exudates and (in)activation of plant defense systems (Carvalhais, Schenk, and Dennis 2017; Lebeis et al. 2015). We previously demonstrated the impact of the plant stress hormone jasmonic acid (JA) on growth and secondary metabolism of streptomycetes, establishing a direct effect on growth and antibiotic production (Chapters 3 and 4). We also showed that the adverse effect of JA on growth was partly neutralized by conjugation of JA with glutamine (Chapter 5). Here we show that plant hormone tolerance greatly varies within the family of *Streptomycetaceae.* While streptomycetes can grow in the presence of 0.5 mM JA, 0.7 mM SA and 0.6 mM IAA, growth of most *Streptacidiphilus* strains was inhibited at these concentrations. LC-MS analyses of *Streptomycetaceae* exposed to JA revealed conjugation of JA to a range of aminoacyl variants, primarily OHJA-Val, OHJA-Phe, OHJA-Ile, OHJA-Leu, OHJA-Tyr and JA-Gln. To our knowledge, this is the first time such diversity of JA conjugation is shown within the family of *Streptomycetaceae*. Most conjugates were found in *Streptomyces* extracts, with JA-Gln being one of the most abundantly detected conjugates among the family of *Streptomycetaceae*. Interestingly, JA conjugated to Gln and Phe could indeed be applied to *Streptacidiphilus* at higher concentrations than JA, which shows that conjugation of JA to either Gln or Phe lowers JA toxicity. Those conjugates might either not enter the cells anymore or they bind less effectively to their putative target(s). In addition, JA was also conjugated to *N*-methyl-Gln by *S. roseifaciens* and the plant-associated strain *S.* sp. Atmos39. Only the amino acid part *N*-methyl-Gln was previously described and detected as a metabolic intermediate in bacteria utilizing monomethylamine as a carbon and/or nitrogen source (Wischer et al. 2015). The amino acid was also found as one of the constituents of green tea extract (Yokogoshi and Kobayashi 1998). To our knowledge, the effect(s) of JA-*N*-methyl-Gln are largely unknown and this compound will be an interesting target for future plant physiology and plant-microbe studies. Noteworthy, the nodes observed for amino acid conjugates of hydroxylated JA (OHJA) were mainly observed in the extracts of the *Streptacidiphilus* isolates, together with some plant associated *Streptomyces* species. Neither the lab strains nor the soil isolates tested were able to produce detectable amounts of Phe, Tyr, Val, Ile or Leu

conjugates of OHJA, suggesting this feature might be attributed to specific groups of microbes within the family of *Streptomycetaceae*.

JA conjugation in plants gives function and specificity to the phytohormone and hydroxylation is believed to deactivate JA and its conjugates (Caarls et al. 2017). Conjugation of JA with amino acids, such as Ile, Leu, Val, Ala, Tyr, and Phe is believed to play a role in the signal transduction pathway of jasmonate-responsive events, like the emission of volatile organic compounds that activate the defense systems in neighboring plants (Piotrowska and Bajguz 2011). However, the JA-Gln conjugate was only reported once in a study that investigated the leaves of *Nicotiana attenuata* plants after treatment with oral secretions of the herbivorous tobacco hornworm, but the function and enzymes responsible for the conjugation to JA-Gln remained unclear (Wang et al. 2007). It is tempting to speculate that the responsible enzymes were not found because the conjugation of JA with Gln was not done by the plant but by members of its endophytic microbiome. However, the enzymes responsible for JA conjugation in *Streptomyces* and *Streptacidiphilus* have not yet been elucidated, which is a first step to validate that microbes may be (in part) responsible for the accumulation of conjugated JA variants in plants. To further establish if indeed the conjugation acts as a resistance mechanism *in vivo*, (over)expression of the responsible enzymes should ensure high tolerance to JA in *Streptacidiphilus*. Phylogenomic analysis of strains with and without acquired JA tolerance could guide us to find those enzymes, as they are likely to conjugate JA more actively and/or effectively (Chapter 5).

We showed that bacteria belonging to the *Streptacidiphilus* genus displayed low plant hormone tolerance. Interestingly, there are very few reports on plant associated *Streptacidiphilus* species. This could be the result of biased isolation techniques that are generally not optimized for acidophilic microbes, but it could also be a result of their intolerance to plant hormones, contributing to their inability to inhabit this niche. Studies aiming at the identification and isolation of plant associated *Streptacidiphilus* strains are required to compensate for this bias.

We failed to improve JA tolerance in *S. coelicolor* or in *Streptacidiphilus* isolates. One explanation may be that JA or its metabolites targets major cellular process(es) and/or component(s), and therefore long-term high

exposure does not enhance JA tolerance. For example, resistance mechanisms against the lantibiotic nisin, which is commonly used as food additive to prevent food spoilage, is hardly observed because it targets an extremely well conserved essential cellular component that cannot readily be modified (Breukink and de Kruijff 2006; Draper et al. 2015). Lantibiotics bind to the pyrophosphate group of lipid II molecule to inhibit cell wall synthesis and subsequently form pores in the membrane (Breukink et al. 1999). Because the pyrophosphate molecule is difficult to be modified by bacteria, resistance to lantibiotics rarely appears. However, as mentioned before, conjugation of JA to JA-Phe and JA-Gln did at least partly detoxify JA. Thus, mechanisms that lower JA toxicity do exist, but enhanced JA-tolerance could not be obtained in our experiments by long-term JA exposure.

Our results further suggested that plant hormones add selective pressure on bacterial communities in soil, rhizosphere or roots. Interestingly, *(Para)Burkholderia* was isolated on media with relatively high concentrations of JA, SA and IAA. The genus *(Para)Burkholderia* has been isolated from a wide range of niches, including humans, animals, soil and plants (Eberl and Vandamme 2016). Molecular signatures and phylogenomic analysis of the genus *Burkholderia* has resulted in a division of this genus into *Burkholderia,* containing pathogenic organisms, and the genus *Paraburkholderia,* harboring mostly environmental species (Sawana, Adeolu, and Gupta 2014). Several *(Para)Burkholderia* species have been reported to fix nitrogen, nodulate legumes and to promote plant growth (Suarez-Moreno et al. 2012). Their ability to successfully inhabit the plant niche may be associated with their tolerance towards plant hormones. Hence, plant hormones might not only indirectly play a role in shaping the plants microbiome by influencing the composition of root exudates and (in)activation of plant defense systems, but also directly by allowing only a subset of bacteria to colonize and enter the root system. Colonization studies with species or strains with variable degrees of hormone tolerance and with mutants that are more sensitive to plant hormones, will be needed to address this hypothesis and to further elucidate if plant hormones have a gate keeping function.

Supplementary figures

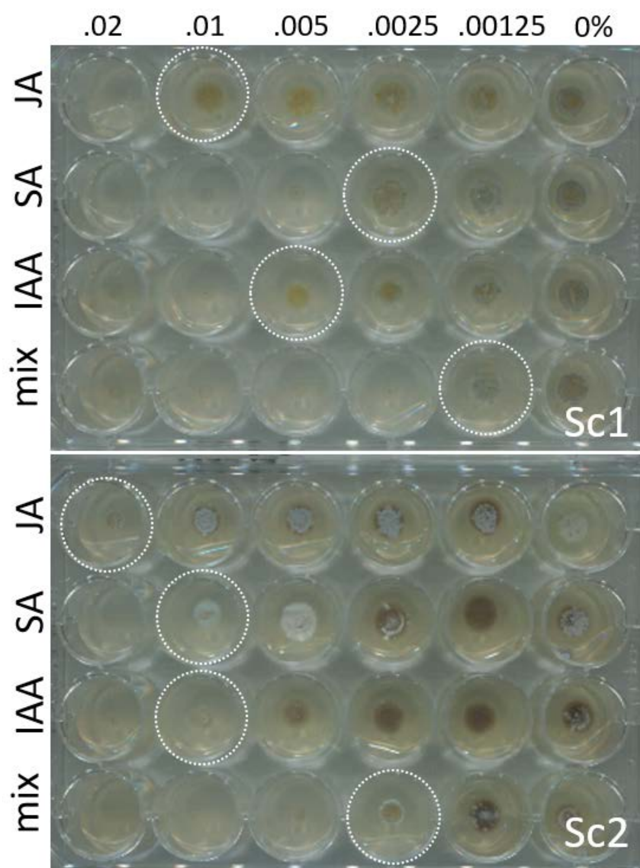

Figure S21: **Plant hormone toxicity is slightly different among *Streptacidiphilus* isolates Sc1 and Sc2.** Sc1 and Sc2 were grown on ½ PDA in 24-wells plates with doubled amount of JA, SA, IAA and a mix of the three from right to left. Dashed circles indicate wells were growth was observed. Sc1, which was shown to be more susceptible to JA, SA and IAA, indeed shows lower tolerance to the plant hormones than Sc2. Note that both strains suffer when all the phytohormones are applied at the same time, starting from 0.0125% w/v, showing an additive toxicity effect when all the plant hormones are administered.

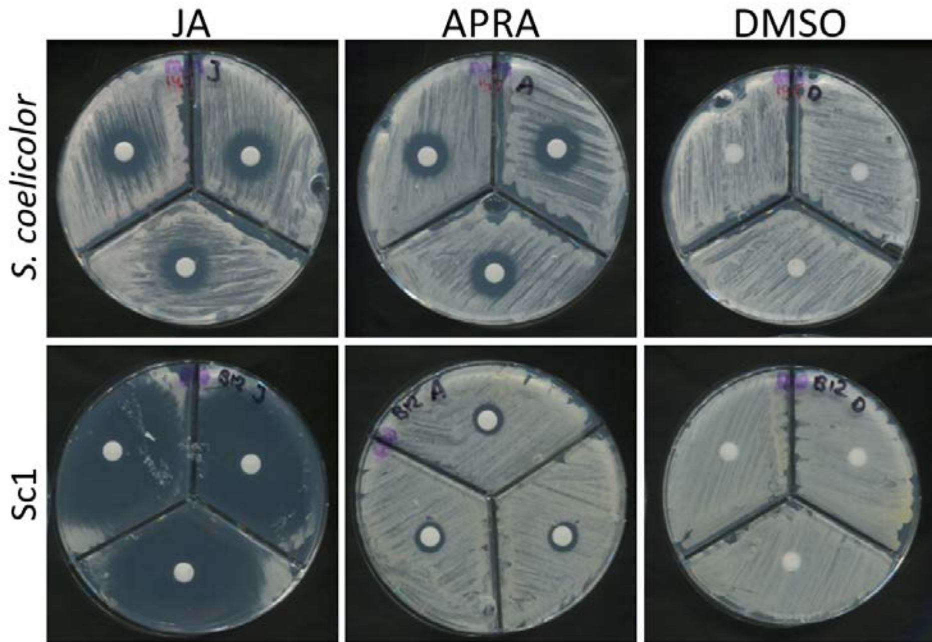

Figure S22: ***Streptacidiphilus* and *Streptomyces* show comparable tolerance towards apramycin and DMSO.** Disks contained 1 mg jasmonic acid (JA), 5 µg apramycin (APRA) or 10 µL DMSO (DMSO). JA and APRA cause a 5 mm zone of growth inhibition in *S. coelicolor*. For *Streptacidiphilus* Sc1 growth is barely visible when JA is applied, but only a 2 mm halo when APRA is applied. No halos are visible when DMSO is applied to the filter disks.

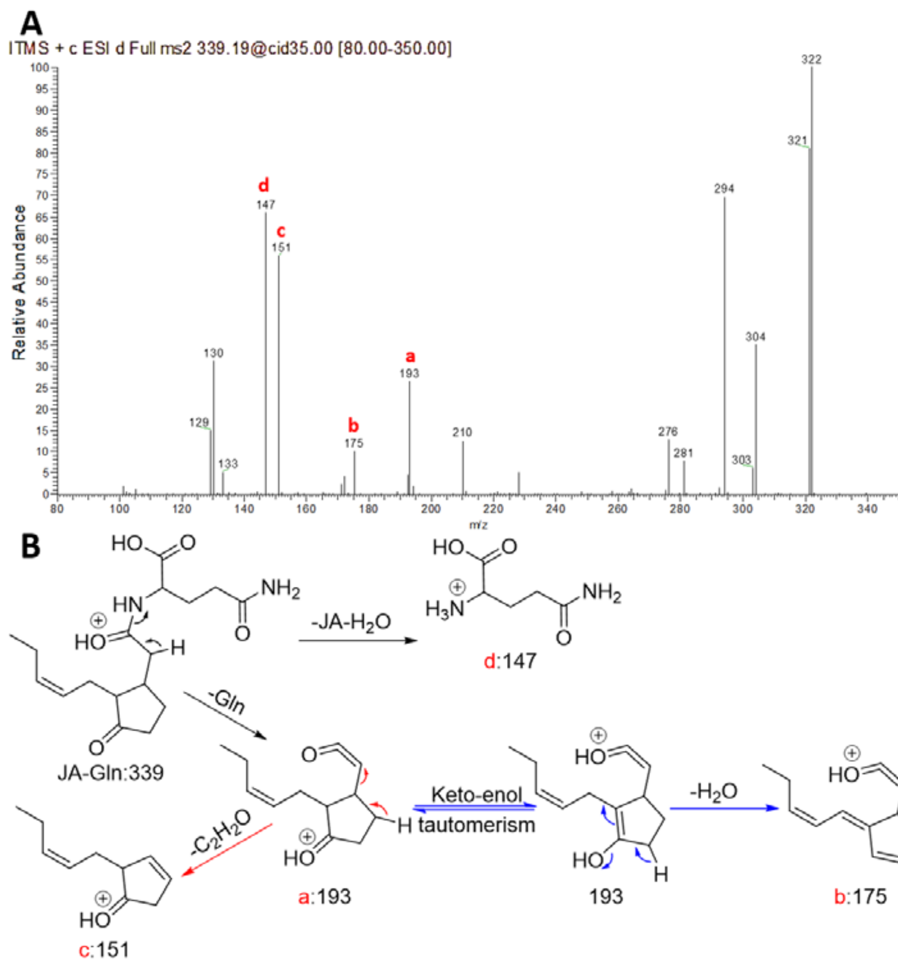

Figure S23: **A**: **MS² spectrum of JA-Gln.** The most characteristic fragments a – d are marked. **B**: **The likely fragmentation pathway of JA-Gln.** Fragmentation resulted in the observed fragments a – d, which are labelled using their nominal masses. The position of the positive charge is arbitrary.

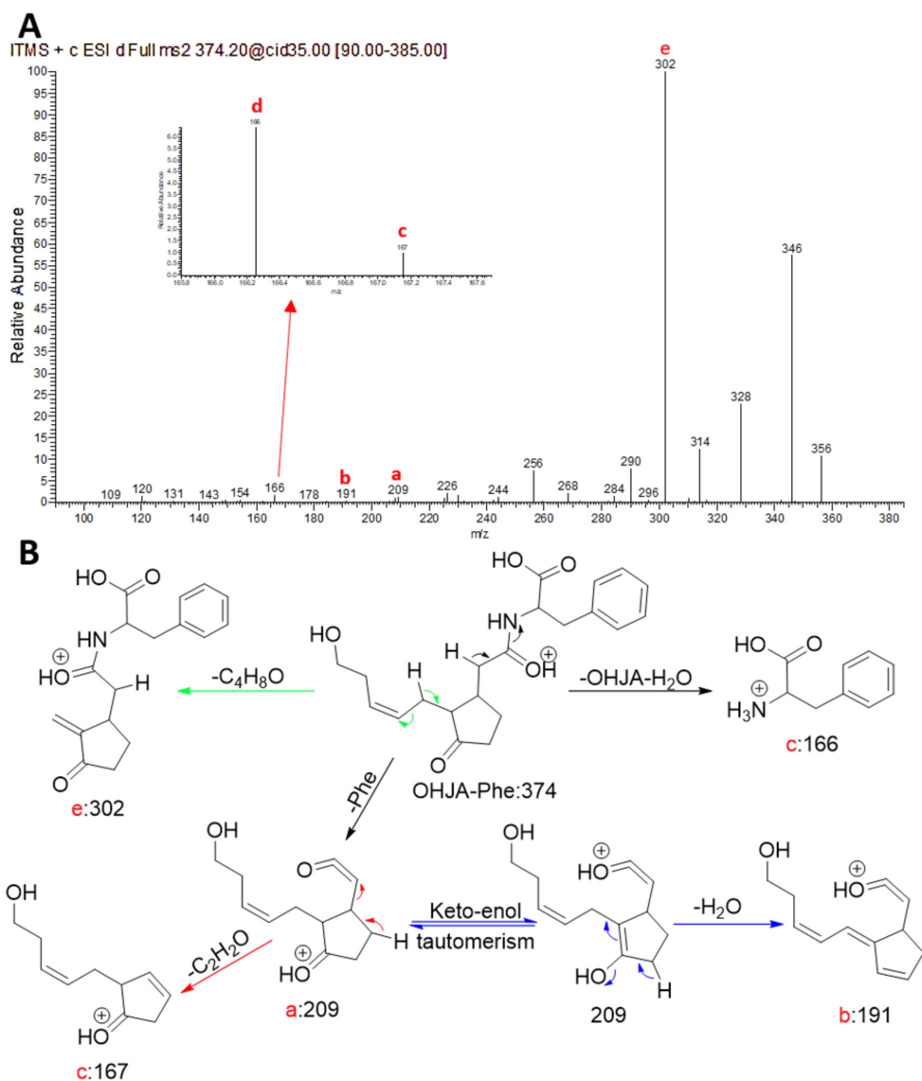

Figure S24: **A**: **MS^2 spectrum of OHJA-Phe.** The most characteristic fragments a – e are marked. **B**: **The likely fragmentation pathway of OHJA-Phe.** Fragmentation resulted in the observed fragments a – e, which are labelled using their nominal masses. The position of the positive charge is arbitrary.

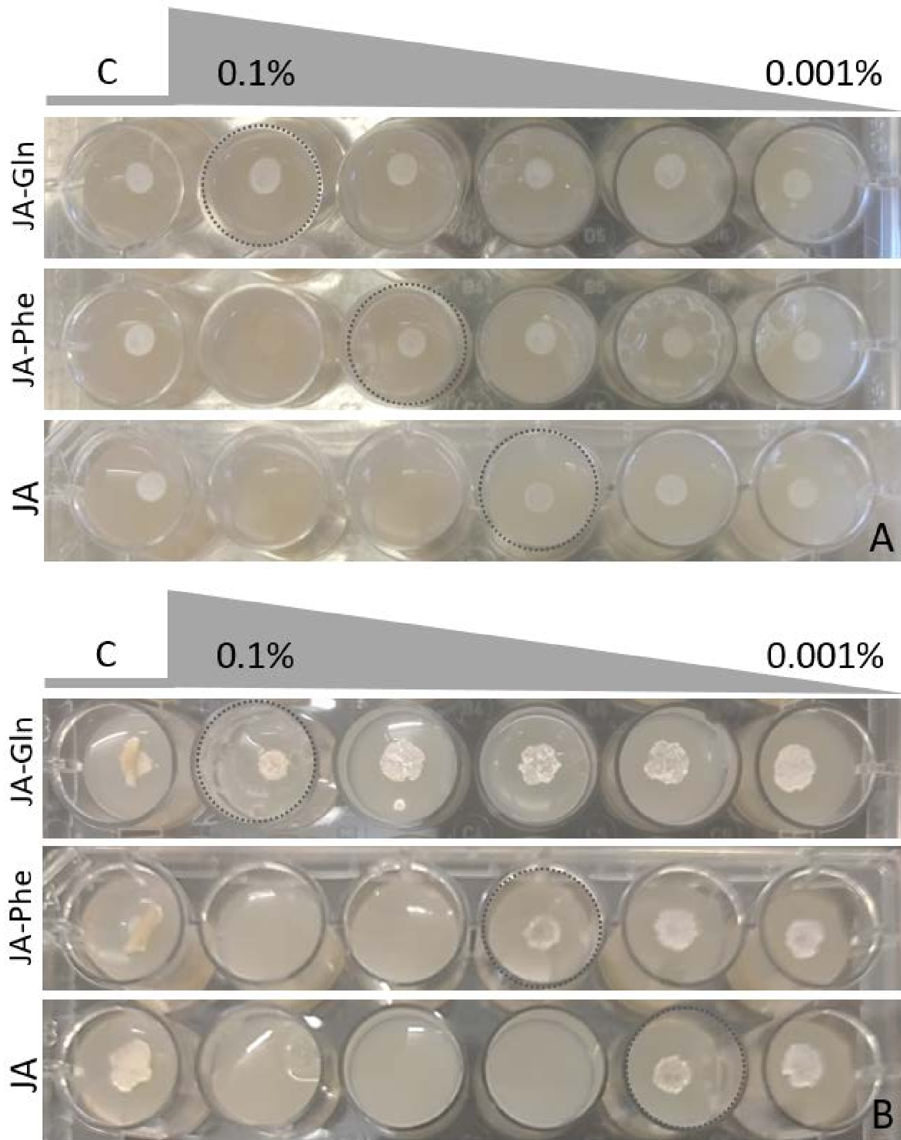

Figure S25: **MIC of JA-Phe and JA-Gln compared to JA.** From left to right, every well represents a 3-fold concentration decrease, resulting in 0.1%, 0.03%, 0.01%, 0.003% and 0.001% w/v addition of the compound indicated on the left (JA-Gln, JA-Phe and JA). A) Representative *Streptacidiphilus* isolate that grows on 0.01% (0.5mM) JA. The isolate still grows on 0.03% (1mM) JA-Phe and 0.1% (3mM) JA-Gln. B) Representative *Streptacidiphilus* isolate that stops growth on 0.01% (0.5mM) JA. The isolate still grows on 0.01% (0.3mM) JA-Phe and even 0.1% (3mM) JA-Gln.

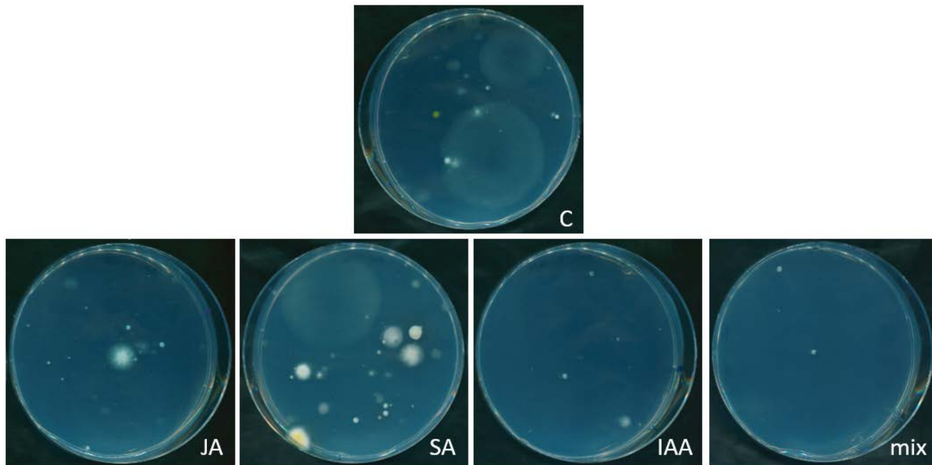

Figure S26: **Indole acetic acid adds selective pressure.** Plating a 10^{-2} dilution of 10 mg/mL soil in MQ suspension on MM (C), MM + 0.01 JA (JA) and MM + 0.01% SA (SA) gives rise to a variety of colony morphologies and ~15 CFUs. Addition of 0.01% IAA (IAA) or a mixture of all three hormones (mix) results in 4 and 3 CFUs respectively.

Supplementary tables

Table S4: **Growth of Streptomyces from the MBT collection on JA, SA, IAA and the three plant hormones mixed.** All strains included could grow on the highest concentration of 5 mM JA, and apart from MBT96, on the highest concentration of 7 mM SA as well. IAA slightly reduces growth in a third of the isolates already at 0.6mM, and half of the isolates fail to grow on 6mM IAA. S = Same growth compared to control. R = reduced growth compared to control. - = no growth.

	JA mM			SA mM			IAA mM		
Strain	0.5	2	5	0.7	4	7	0.6	3	6
MBT1	S	S	R	S	S/R	R	-	R	-
MBT84	S	S	S	S	R	S	-/R	R	-/R
MBT27	S	S	S	S	S/R	R	-	R	-/R
MBT91	S	S	R	S	S	S/R	S	S/R	R
MBT76	R	R	R	R	R	R	-	-	-
MBT62	S	R	S/R	S	S/R	R	R	R	R
MBT42	S	S	R	R	R	R	R	R	R
MBT21	R	R	R	R	R	R	-	-	-
MBT16	S	R	R	R	R	R	R	R	R
MBT5	S	R	R	S	S/R	R	S	S/R	R
MBT92	S	R	R	S	S	R	R	-/R	-
MBT96	S/R	S/R	S/R	S	R	R/-	R	-	-/R
MBT47	S	S	S/R	S	S	S/R	R	R/-	-/R
S. lividans	S	S/R	S/R	R	S/R	R	R	R	R
S. coelicolor M145	S	S	S/R	S	S	S	R	R	R
Total surviving	15	15	15	15	15	14	10	10	7