Investigating the role of exudates in recruiting *Streptomyces* bacteria to the *Arabidopsis thaliana* root microbiome

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Abstract. *Arabidopsis thaliana* has a diverse but consistent root microbiome, recruited in part by the release of fixed carbon in root exudates. Here we focussed on the recruitment of *Streptomyces* bacteria, which are well established plant-growth-promoting rhizobacteria and which have been proposed to be recruited to *A. thaliana* roots by the release of salicylic acid. We generated high quality genome sequences for eight *Streptomyces* endophyte strains and showed that although some strains do enhance plant growth, they are not attracted to, and do not feed on, salicyclic acid. We used $^{13}CO_2$ DNA-stable isotope probing to determine which bacteria are fed by the plants in the rhizo- and endosphere and found that streptomycetes did not feed on root exudates *in vivo*, despite the fact that they can use exudate as sole carbon and nitrogen sources *in vitro*. We confirmed increased root colonisation by streptomycetes in plants that constitutively produce salicylic acid, but these plants exhibited a pleiotropic phenotype of early senescence and weak growth. We propose that streptomycetes are attracted to the rhizosphere by root exudates but can be outcompeted for this food source by more abundant proteobacteria and most likely feed off unlabelled complex organic matter.

Introduction. Plants form stable and beneficial interactions with bacteria at all stages of their life cycle, but we have a limited understanding of how these interactions are directed or maintained. Young plants can release 30-40% of photosynthetically fixed carbon into the soil as root exudates and these help shape the plant microbiome (Haichar et al 2016, Whipps 1990). Exudates contain a diverse range of compounds, many of which may be metabolized by beneficial bacterial communities (Bais et al 2006, Zhalnina et al 2018). Understanding the signals that recruit different bacterial taxa is economically important because plant-growth-promoting rhizobacteria (PGPR) can increase crop growth and protect against disease (Berg 2009). There is growing interest and investment in biological solutions to crop loss, including the application of beneficial microbes as seed coatings and supplements to agricultural soils (Berg 2009, Wei and Jousset 2017). Increasing the presence of antibiotic-producing bacteria such as *Streptomyces* species in agricultural soils has been shown to suppress fungal pathogens in numerous recent studies, (e.g. Cha et al 2016, Inderbitzin et al 2018, Ren et al 2018, Zhou et al 2018) and some *Streptomyces* endophyte species are marketed as fungal growth suppressors (Chaurasia et al 2018, Rey and Dumas 2017, Viaene et al 2016).

Streptomycetes are saprophytic bacteria that play an important ecological role as composters in soil and as prolific producers of antibiotics (van der Meij et al 2017). They evolved filamentous growth ca. 440 mya when plants were first colonising land and it has been suggested that this enabled them to successfully colonise plant roots (Chater 2016). Several studies have reported that streptomycetes are abundant inside the roots of the model plant *Arabidopsis thaliana* (Bai et al 2015, Bodenhausen et al 2013, Bulgarelli et al 2012, Carvalhais et al 2015, Lebeis et al 2015, Lundberg et al 2012, Schlaeppi et al 2014), and others have shown that streptomycetes can protect crop plants such as strawberry, lettuce, rice, and wheat against biotic and abiotic stressors, including drought, salt stress, and fungal pathogens (see Schrey and Tarkka 2008, Viaene et al 2016 for recent reviews). Recent studies have reported that the proportion of *Streptomyces* bacteria in the *A. thaliana* rhizosphere and roots is positively correlated with the levels of phenolic compounds released in *A. thaliana* root exudates, including the defence hormone salicylic acid (SA) (Badri et al 2013, Carvalhais et al 2015, Lebeis et al 2015). It was also reported recently that some phytohormones induce the production of antimicrobial compounds by endophytic streptomycetes (van der Meij et al 2018). Not all plant species enrich streptomycetes in their roots, a notable example being barley, and this suggests there are plant-specific signals that shape the root microbiota (Bulgarelli et al 2012, Bulgarelli et al 2015, Haichar et al 2008).

In this study we set out to identify which root and rhizosphere bacteria feed off *A. thaliana* root exudates and to test the previously published hypothesis that *Streptomyces* bacteria use plant-produced SA as a sole carbon source (Lebeis et al 2015). We report that *Streptomyces* endophytes and other *Streptomyces* strains can indeed grow *in vitro* using *A. thaliana* root exudates as a sole carbon and nitrogen source, but *in vivo*, DNA stable isotope probing (DNA-SIP) revealed that *Streptomyces* were not feeding off root exudates in the rhizosphere or the endophytic compartment. We also show that, although root colonisation by *Streptomyces* endophyte isolates increases in *A. thaliana cpr5* plants (which constitutively produce SA) as has been reported previously (Lebeis et al 2015), these isolates were not attracted to SA and could not grow using SA as a sole carbon source. We conclude that colonisation was likely influenced by the pleiotropic effects of the *cpr5* mutation.

Materials and Methods.

Isolation of *Streptomyces* endophytes. Wild-type *A. thaliana* Col-0 seeds were sterilised by washing in 70% (v/v) ethanol for 2 minutes, 20% (v/v) sodium hypochlorite for 2 minutes, then five times in sterile water. Individual seeds were sown into pots of sieved Levington F2 compost, placed at 4°C for 24 hours, then grown for 4 weeks under conditions of 12 hours light / 12 hours dark at 22°C. Plants were taken aseptically from pots and roots tapped firmly to remove as much adhering soil as possible. Root material was then placed into sterile PBS-S buffer (PBS-S: 6.33 g $NaH_2PO_4.H_2O$, 16.5 g $Na_2HPO_4.H_2O$ dissolved in 1L dH_2O, to which 200 µl Silwet L-77 was added after autoclaving) for 30 minutes on a shaking platform. Roots were placed into fresh PBS-S and washed for 30 minutes before removing any remaining soil particles with sterile tweezers. Cleaned roots were transferred to 25ml fresh PBS-S and sonicated for 20 minutes in a sonicating water bath to remove any remaining material still attached to the root (as described in Bulgarelli et al 2012). The roots were crushed in sterile 10% (v/v) glycerol and serial dilutions were spread onto either soya flour mannitol (SFM) agar, starch casein agar, or minimal medium agar containing sodium citrate (Lebeis et al 2015). Plates were incubated at 30°C for up to 14 days. Colonies resembling streptomycetes were re-streaked onto SFM agar and identified by 16S rRNA gene PCR amplification and sequencing with universal primers PRK341F and MPRK806R (see Table S1 for primers, plasmids and strains used in this work). *Streptomyces* strains were maintained on SFM agar (N1, N2, M2, M3 and *S. coelicolor* M145), Maltose/Yeast extract/Malt extract (MYM) agar with trace

elements (L2) or ISP2 agar (*S. lydicus* strains); media recipes are detailed in Table S2. Spore stocks were made as described previously (Kieser et al 2000).

Genome sequencing and analysis. High quality genome sequences were obtained for newly isolated strains N1, N2, M2, M3, and L2, as well as three known strains of *Streptomyces lydicus;* one isolated from the commercial plant growth-promoting product Actinovate and two additional *S. lydicus* strains (ATCC25470 and ATCC31975) obtained from the American Type Culture Collection. Strains were sequenced using PacBio RSII sequencing technology at the Earlham Institute, Norwich, UK, (as described in Holmes et al 2018). Biosynthetic gene clusters were predicted using antiSMASH 4.0, and genomes were annotated using RAST (Aziz et al 2008, Weber et al 2015). Amino acid sequences were uploaded to the KEGG Automatic Annotation Server (KAAS) for functional annotation of genes and metabolic pathway mapping. To identify whether isolates carried homologues to known salicylic acid (SA) degradation genes, all experimentally verified pathways involving SA (or salicylate) degradation were identified using the MetaCyc database (http://www.metacyc.org/). Amino acid sequences of characterised genes involved in each of the five pathways were then retrieved from UniProt and used to perform Blast searches against predicted ORFs for each streptomycete strain. The results of the best hit (% identity and % query coverage) are given in Table S3.

Antibiotic bioassays. *Streptomyces* spores (4 µl of 10^6 spores ml^{-1}) were pipetted onto the centre of agar plates and incubated at 30 °C for 7 days before adding indicator strains. *Candida albicans*, *Bacillus subtilis*, a clinical isolate of Methicillin Resistant *Staphylococcus aureus* isolated from a patient at the Norfolk and Norwich University Hospital UK (Qin et al 2017), *Escherichia coli* and *Pseudomonas syringae* DC3000 were grown overnight in 10 ml Lysogeny Broth (LB) (1% tryptone, 0.5% NaCl, supplemented with 0.1% glucose for *P. syringae*). These were sub-cultured 1 in 20 (v/v) for a further 4 hours at 30°C for *P. syringae* and 37°C for *E. coli*. These cultures were then used to inoculate 100 ml of molten LB (0.5% agar, plus 0.1% glucose for *P. syringae*), of which 3 ml was used to overlay agar plates containing *Streptomyces* colonies. Plates were incubated for 48 hours at 30°C, and bioactivity was indicated by a clear halo around the *Streptomyces* colony. For bioassays using the fungal strains *Lomentospora prolificans* or *Gaeumannomyces graminis*, *Streptomyces* species were grown for 7 days, and then a plug of the fungus (grown on potato glucose agar, Sigma Aldrich, for 14 days) was

placed at the edge of the agar plate. Plates were incubated at 25°C for up to 14 days to assess inhibition of fungal growth. Bioassays were carried out on a range of different media (Tables S2, S4).

***In vitro* Plant growth promotion assays**. *A. thaliana* col-0 seeds were sterilised and plated onto Murashige and Skoog (MS) medium (Duchefa Biochemie, Harlem, Netherlands) (4.43 g L^{-1} in dH_2O, supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar) which were then left at 4 °C in the dark for 24 hours before being placed, vertically, under long day growth conditions (12 h light / 12 h dark) at 22°C for 10 days. Seedlings were then transferred to square agar plates containing MS agar (1.5% agar, 0% sucrose) and allowed to equilibrate, vertically, overnight at 22 °C. *Streptomyces* spores were diluted to 10^6 spores ml^{-1}, and 1 µl of this solution was added to the top of the root system of each seedling and allowed to dry. 16 replicate seedlings were inoculated per *Streptomyces* strain. 10% (v/v) glycerol was added to control seedlings. Plates were grown vertically for 16 days, 12 h light/12 h dark at 22°C before measuring plant biomass (dry weight). The biomass of plants with different inocula were compared via ANOVA and Tukey's Honestly Significant Difference (HSD) tests; biomass was log-transformed to ensure normality.

IAA production assays *Streptomyces* isolates were grown on cellophane membranes covering YMD media (Table S2) supplemented with 5 mM tryptophan. After 7 days, cellophane membranes with bacterial biomass were removed and plates were flooded with Salkowskis reagent (as in Bric et al 1991). A red colour indicates that IAA has leached into the medium.

Collection of root exudates. Seeds were sterilised as above and placed at 4°C for 24 hours before being grown, vertically, in conditions of 12 h light/12 h dark for 7 days at 22°C on MS agar. Seedlings were transferred to 12 well plates, containing 3 ml of liquid MS (0% sucrose) in each well and then grown for 10 days before being washed and transferred to new wells containing 3 ml sterile water for 5 days. After removing plant material, liquid from each well was filter-sterilised and added to sterile agarose (0.8% w/v) to make solid growth medium plates. Spores of each *Streptomyces* isolate were streaked onto these plates and incubated for 7 days at 30°C. Agarose/water (0.8% w/v) plates were used as a control to assess growth on root exudates.

Stable Isotope Probing. Sterilised *A. thaliana* seeds were sown singly in pots containing 100 ml sieved Levington F2 compost, soaked with dH_2O. These were placed in the dark at 4°C for 48 hours, then transferred to short-day growth conditions (8 h light/16 h dark) at 22°C for 32 days before exposure to CO_2. Each plant was placed in air-tight, transparent 1.9 L cylindrical tubes. Three plants were exposed to 1000 ppmv of $^{12}CO_2$ and three plants were exposed to 1000 ppmv of $^{13}CO_2$ (99%, Cambridge isotopes, Massachusetts, USA). Three unplanted controls containing only Levington F2 compost were additionally exposed to 1000 ppmv of ^{13}C labelled CO_2 to control for autotrophic consumption of CO_2 by soil microbes. CO_2 treatments took place over a period of 21 days (ending 53 days after germination). CO_2 was manually injected into tubes every 20 minutes over the course of the 8 hour light period, to maintain the CO_2 concentration at ~1000 ppmv. The volume of CO_2 to be added at each injection was determined by measuring the rate of uptake of CO_2 over 20 minutes every 4 days. CO_2 was measured using an Agilent 7890A gas chromatography instrument with a flame ionisation detector and a Poropak Q (6ft x 1/8") HP plot/Q (30 m x 0.530 mm, 40 μm film) column with a nickel catalyst and a nitrogen carrier gas. The instrument was run with the following settings: injector temperature 250°C, detector temperature 300°C, column temperature 115°C and oven temperature 50°C. The injection volume was 100 μl and the run time was 5 mins, with CO_2 having a retention time of 3.4 mins. Peak areas were compared to a standard curve (standards of known CO_2 concentration were prepared in 120 ml serum vials that had been flushed with 80%/20% nitrogen-oxygen mixture). At the end of the light period each day, tube lids were removed to prevent the build-up of respiratory CO_2 during the dark period. Just before the next light period, tubes were flushed with an 80%/20% nitrogen-oxygen mix to remove any residual CO_2 before replacing the lids and beginning the first injection of 1.9 ml CO_2.

Sampling and DNA extraction from CO_2 incubated soil, rhizosphere, and roots. Two samples of root-free "bulk soil" were collected from each planted and unplanted pot; samples were snap-frozen in liquid nitrogen and stored at -80°C. For the planted pots, roots were tapped until only soil firmly adhering to the root surface remained; the remaining soil was defined as the rhizosphere fraction. To collect this, roots were placed in 25 ml sterile, PBS-S buffer (see above) and washed on a shaking platform at top speed for 30 min before transfer to fresh PBS-S. Used PBS-S from the first washing stage was centrifuged at 3200 x g for 15 minutes, and the supernatant was removed. The resulting pellet (the rhizosphere sample) was snap-frozen

and stored at -80°C. The roots were then shaken in fresh PBS-S for a further 30 minutes before removing any remaining soil particles with sterile tweezers. The cleaned roots were finally transferred to fresh PBS-S and sonicated for 20 minutes in a sonicating water bath (as described in Bulgarelli et al 2012). The root ("endophytic") sample for each plant was then separated into two samples, and snap frozen and stored at -80°C. The endophytic sample consisted of bacteria within the roots and those very firmly attached to the root surface (the "rhizoplane"). A modified version of the manufacturer's protocol for the FastDNA™ SPIN Kit for Soil (MP Biomedicals) was used to extract DNA from soil, rhizosphere, and root samples. Modifications included pre-homogenisation of the root material by grinding in liquid nitrogen before addition of lysis buffer, an extended incubation time (10 minutes) in DNA matrix buffer, and elution in 150 µl of sterile water. DNA yields were quantified using a Qubit fluorimeter.

Density gradient ultracentrifugation and fractionation. DNA samples from the rhizosphere, roots, and unplanted soil were subjected to caesium chloride (CsCl) density gradient separation using an established protocol (Neufeld et al 2007). For each of the replicate rhizosphere samples from both the $^{12}CO_2$ and $^{13}CO_2$ incubated plants, 1.5 µg of DNA was loaded into the CsCl solution with gradient buffer. For the three unplanted soil sample replicates, 1 µg of DNA was used. For the three root sample replicates under each of the planted treatments ($^{12}CO_2$ and $^{13}CO_2$), it was necessary to combine the three replicates as the low DNA yields from each sample did not meet the minimum DNA yield requirement (0.5 µg) for ultracentrifugation on their own. Thus, 0.2 µg of DNA was pooled from each of the three replicates per $^{12}CO_2$ and $^{13}CO_2$ treatment, and the final 0.6 µg was loaded into the CsCl solution. After ultracentrifugation, the density of each fraction was measured using a refractometer in order to check for successful gradient formation. DNA was precipitated from fractions as detailed in Neufeld et al 2007 and stored at -20°C before use as a template in qPCR and PCR reactions.

Fraction selection via qPCR and 16S rRNA gene amplicon sequencing. To identify fractions containing heavy (^{13}C) and light (^{12}C) DNA for each sample, 16S rRNA gene copy number was quantified across fractions using qPCR. Reactions were carried out in 25 µl volumes. 1 µl of template DNA (either sample DNA or standard DNA), or dH_2O as a control, was added to 24 µl of reaction mix containing 12.5 µl of the 2x Sybr Green Jumpstart Taq Ready-mix (Sigma Aldrich), 0.125 µl each of the primers 341F 806R, 4 µl of 25 mM $MgCl_2$,

0.25 µl of 20 µg/µl Bovine Serum Albumin, and 7 µl dH_2O. Sample DNA, standards (a dilution series of the target 16S rRNA gene at known quantities), and negative controls were quantified in duplicate. Reactions were run under the following conditions: 96°C for 10 mins; 40 cycles of 96°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min; 96°C for 15 sec; 100 cycles at 75°C-95°C for 10 secs, ramping 0.2°C per cycle. Reactions were performed in 96-well plates (Bio-Rad). The threshold cycle (C_T) for each sample was then converted to target molecule number by comparing to C_T values of a dilution series of target DNA standards. Following quantification of 16S rRNA gene number in each fraction, fractions spanning the peaks in 16S rRNA gene copy number were identified. Equal quantities of each fraction were combined to create a "heavy" buoyant density (labelled) and "light" buoyant density (unlabelled) fraction for each sample, respectively. The 16S rRNA genes were amplified for each of these fractions using the Universal primers PRK341F and MPRK806R (Table S1), and the resulting PCR product was purified and submitted for 16S rRNA gene amplicon sequencing using an Illumina MiSeq at Mr DNA (Molecular Research LP), Shallowater, Texas, USA. Sequence data were then processed at Mr DNA using their custom pipeline (Dowd et al 2008a, Dowd et al 2008b). As part of this pipeline, pair-end sequences were merged, barcodes were trimmed, and sequences of less than 150 bp and/or with ambiguous base calls were removed. The resulting sequences were denoised, and OTUs were assigned by clustering at 97% similarity. Chimeras were removed, and OTUs were assigned taxonomies using *BlastN* against a curated database from GreenGenes, RDPII, and NCBI (DeSantis et al 2006). Plastid-like sequences were removed from the analysis. All data received from Mr DNA were then statistically analysed using *R* 3.2.3 (2015). All the 16S rRNA gene amplicon sequences have been submitted to the European Nucleotide Archive (ENA) database under the study accession number PRJEB30923.

Generating eGFP-labelled *Streptomyces* strains. Plasmid pIJ8660 (Table S1, John Innes Centre, Norwich, UK) containing an optimised eGFP gene under the control of the constitutive *ermE** promoter and the *aac* apramycin resistance marker (Sun et al 1999) was conjugated into *Streptomyces* strains (as described in Kieser et al 2000). Exconjugants were selected and maintained on SFM agar plates containing 50 µg ml^{-1} apramycin. For confocal microscopy, *Arabidopsis thaliana* Col-0 seeds were germinated as described for plant growth promotion assays and grown vertically at 22 °C for 9 days under a 12-hour photoperiod. Seedlings were then transferred to MS (1.5% agar, 0% sucrose) and were allowed to equilibrate for 24 hours

before being inoculated with 1µl of spore suspension (10^6 cfu ml^{-1}) of either M3 or *Streptomyces coelicolor* M145 (both eGFP-tagged). Inoculated seedlings were then left to grow for 3 days before being washed in a 20% (v/v) solution of glycerol containing 1µg ml^{-1} SynaptoRed™ for 10 minutes. A 20 mm section of root (taken from the base of the petiole) was then mounted onto a slide with 100 µl of the SynaptoRed™/glycerol solution. Samples were imaged using a Zeiss LSM510 META laser-scanning confocal microscope with a PlanApochromat 63x (1.4 NA) objective. Green fluorescent protein was excited at 488 nm and emission collected through a 527.5 ± 22.5 nm bp filter, and FM4-64 excited at 543 nm and emission collected through a 587.5 ± 27.5 nm bp filter.

Plant colonisation assays in soil. Wild-type *A. thaliana* Col-0, *cpr5*, *pad4*, and *sid2-2* were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Seeds were sterilised and placed in 500 µl of solutions containing 10^6 pregerminated *Streptomyces* spores ml^{-1} (either *Streptomyces coelicolor* M145 or isolate M3, both eGFP-tagged) in 2xYT (Table S2, Kieser et al 2000). Spores were pre-germinated at 50°C for 10 minutes. 2xYT was used as a control. Seeds were incubated in the solutions for 90 minutes on a rotating shaker before being transferred to pots of sieved Levington F2 compost, soaked with dH_2O. Another 1 ml of pre-germinated spores (or 2xYT control) was pipetted into the soil to a depth of approximately 2 cm below the seed. Pots were then incubated at 4°C for 24 hours, then grown for 4 weeks under a photoperiod of 12h light/ 12h dark at 22°C. Six replicate plants were grown for each plant and streptomycete genotype.

Re-isolation of eGFP-tagged bacteria from roots. Root samples were cleaned as described above and crushed in 1 ml of sterile 10% (v/v) glycerol. 100 µl of the homogenate was plated onto three replicate SFM agar plates containing 50 µg ml^{-1} apramycin as well as 5 µg ml^{-1} nystatin to inhibit fungal growth. Agar plates were incubated at 30°C for 5 days, then colony forming units (CFU) were counted. CFU counts were converted to CFU per gram of root tissue and log-transformed to normalise residuals. Data were analysed via ANOVA and TSD tests.

Testing for sole use of carbon and nitrogen sources. *Streptomyces* strains were streaked onto minimal medium agar plates (Kieser et al 2000) supplemented with either their preferred carbon source as a positive control (5 g ml^{-1} of either mannitol, maltose or sucrose), 3.875 mM sodium citrate, or 0.5 mM SA as a carbon source. Plates with no carbon source were used as a

control. All plates were incubated for seven days at 30°C. To test for the use of 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source, strains were streaked onto Dworkin and Foster medium (Dworkin and Foster 1958) in which 0.2% (w/v) NH_4SO_4 or 0.051% (w/v) ACC was added as a sole nitrogen source, or no nitrogen source as a control. Plates were left for 10 days at 30°C before imaging.

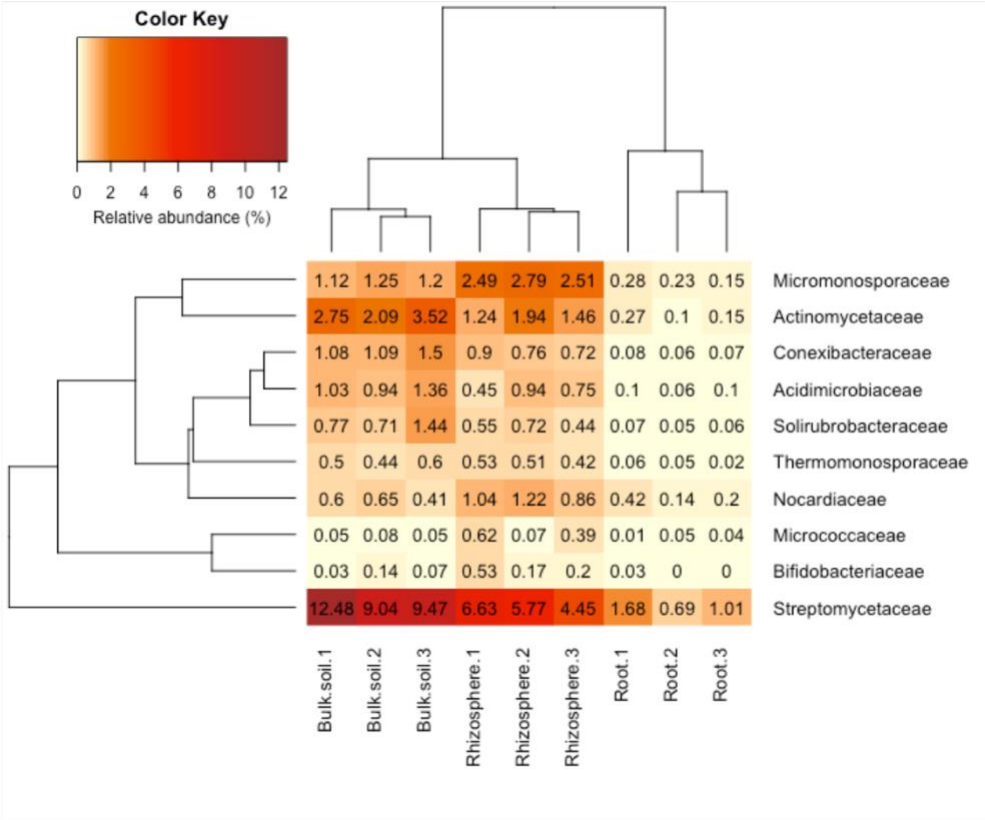
Salicylic Acid (SA) as a chemoattractant. 4 µl of *Streptomyces* spores (10^6 spores ml^{-1}) were placed in the centre of SFM agar plates and 40 µl of either 1 mM or 0.5 mM filter sterilised SA was inoculated onto 6mm filter paper discs (Whatman) and allowed to dry. Discs were then added to one side of the agar plate, 2 cm from the streptomycete spores. SA solutions were prepared by diluting a 100 mM stock solution to a 1 mM or 0.5 mM SA solution in PBS. The 100 mM stock solution was made by dissolving 0.138 g of SA in 2 ml of 100% DMSO before making the solution up to 10 ml with PBS. Thus, the resulting 1 mM and 0.5 mM solutions had a final concentration of 0.2% and 0.1% DMSO, respectively. To check that any observations were not due to the effects of DMSO, control plates were also run alongside the SA experiment, in which discs were soaked in 40 µl of a 0.2% DMSO (in PBS), equivalent to the final concentration of DMSO in the 1 mM SA solution. Plates were incubated for seven days at 30 °C.

Enumeration of bacteria from soil microcosms after exogenous application of salicyclic acid. Levington F2 seed and modular compost (4 ml) was placed into each compartment of a 12-well plate and soaked with 0.5 ml of sterile dH_2O or 0.5 mM SA. Each well was then inoculated with 10^7 spores ml^{-1} solution of either eGFP-tagged M145 or M3, suspended in dH_2O or 0.5 mM SA. Spores of eGFP-tagged M3 or M145 were used to align with the *in vivo* plant colonisation experiments. There were nine replicate wells for each treatment. Well-plates were placed under a photoperiod of 12 h light/12 h dark for 10 days. 100 mg of soil from each well was then diluted in 900 µl of water and vortexed. Serial dilutions were then plated onto SFM containing 50 µg ml^{-1} apramycin (for selection), 10 µg ml^{-1} nystatin, and 100 µg ml^{-1} cyclohexamide (to repress fungal growth). CFU of the *Streptomyces* inoculum were then enumerated on the 10^{-2} dilution plates after 4 days to assess whether SA affected growth in soil.

Results

Bacterial 16S rRNA gene profiling of the *A. thaliana* rhizosphere and endosphere. Several independent studies have reported that *A. thaliana* plants have relatively stable and consistent root bacterial communities, with the enrichment of Actinobacteria within plant roots being predominantly driven by the presence of the family Streptomycetaceae (Bai et al 2015, Bodenhausen et al 2013, Bulgarelli et al 2012, Carvalhais et al 2015, Lebeis et al 2015, Lundberg et al 2012, Schlaeppi et al 2014). To test this on plants grown in our laboratory, we repeated the bacterial 16S rRNA gene profiling using the universal primers PRK341 and MPRK806 (Yu et al 2005) and found the results were in agreement with published studies, in that Streptomycetaceae were the most abundant family of Actinobacteria found in the both the rhizosphere and endophytic compartments (Fig. 1). Although they were not significantly enriched in either compartment compared to the surrounding soil, Streptomycetaceae made up 5.62% (± 1.10% standard deviation) of the rhizosphere community and 1.12% (± 0.50%) of the endophytic community; Actinobacteria as a whole made up 15.15% (± 1.47%) and 2.87% (± 0.88%) of these compartments, respectively (Fig. 1 and S1).

Figure 1. The relative abundance of Actinobacterial families in soil, rhizosphere and root compartments. N = 3 replicate plants in individual pots. Streptomycetaceae was the most abundant family of Actinobacteria in all three compartments. Clustering represents Bray Curtis dissimilarities.



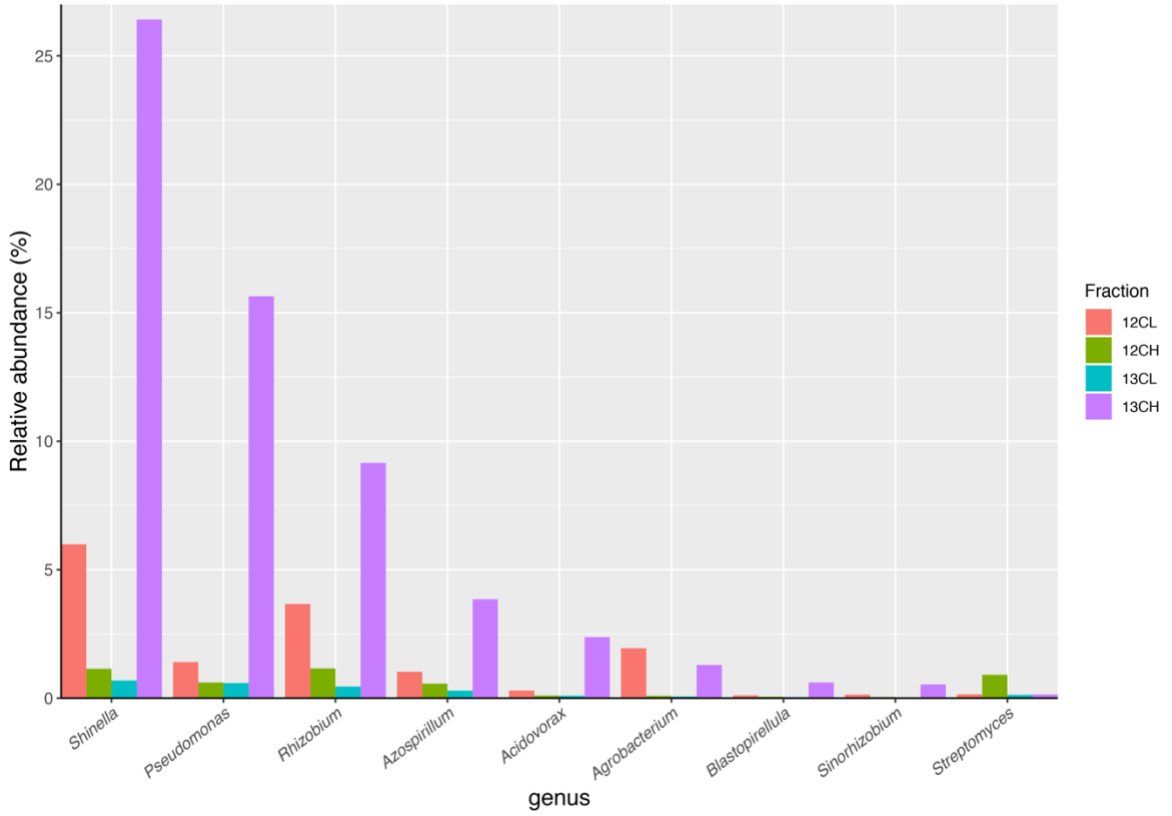
DNA Stable Isotope Probing of the *A. thaliana* rhizosphere and endosphere. To identify which bacteria feed on root exudates in the rhizo- and endosphere of *A. thaliana*, plants were labelled by growing them (n=3) for 21 days under a short-day photoperiod (8 hours light) in the presence of $^{12}CO_2$ or $^{13}CO_2$ in sealed growth chambers (Fig. S2). Unplanted pots were also incubated with $^{13}CO_2$ to control for autotrophic metabolism. Given the relatively short time frame of CO_2 exposure, the ^{13}C was expected to be incorporated mainly into plant metabolites rather than into plant cell wall material. Bacteria that incorporate ^{13}C into their DNA are therefore likely to be feeding on exuded plant metabolites or directly fixing $^{13}CO_2$ autotrophically. After 21 days, total DNA was extracted from the unplanted soil, rhizosphere, and endosphere compartments of the $^{12}CO_2$ or $^{13}CO_2$ incubated plants, and the heavy (^{13}C) and light (^{12}C) DNA were separated by density gradient ultracentrifugation for the rhizosphere and endosphere compartments (Neufeld et al 2007). Heavy and light fractions of the gradient were used for 16S rRNA gene amplicon sequencing using the universal bacterial 16S rRNA primers PRK341 and MPRK806. For the rhizosphere and endophytic samples, bacterial genera were defined as metabolisers of root exudates if they showed at least a two-fold increase in relative abundance in the heavy fraction of $^{13}CO_2$ plants (^{13}CH) compared to both the $^{13}CO_2$ light fraction (^{13}CL) and $^{12}CO_2$ heavy fraction (^{12}CH), respectively. Importantly, the abundance of

these bacteria was also less than two-fold greater in the heavy versus the light fractions of $^{13}CO_2$ unplanted controls which means they are not autotrophically fixing CO_2.

Following sorting of the data and the removal of autotrophs (Table S5), 28 genera showed an average of two-fold (or more) enrichment in the ^{13}CH fraction of rhizosphere samples (Fig S3, Table S5). The majority of these taxa were in the phylum Proteobacteria (24 out of 28 genera), with only one representative each from the phyla Chloroflexi (*Levilinea*), Firmicutes (*Pelotomaculum*), Cyanobacteria (*Chroococcidiopsis*), and the Planctomycetes (*Pirellula*). The most enriched genus was *Pseudomonas*, which demonstrated a 64-fold enrichment in relative abundance between the ^{13}CH (8.63% average relative abundance) and ^{13}CL (0.13% average relative abundance) fractions and a 23-fold enrichment between the ^{13}CH fraction compared to the ^{12}CH control (0.37% relative abundance). A total of 27 genera demonstrated enrichment in the ^{13}CH fraction of the endophytic compartment (Fig 2, Table S5). Here, the majority of root exudate metabolisers were also Proteobacteria (21 genera in total), with three genera belonging to the phylum Planctomycetes (*Blastopirellula*, *Pirellula* and *Gemmata*), one to the Firmicutes (*Clostridium*), one to the Chloroflexi (*Chloroflexus*), and one to the Actinobacteria (*Jatrophihabitans*). The most abundant genus in the ^{13}CH fraction of the endophytic samples was *Shinella*, which demonstrated a 38-fold enrichment between the ^{13}CH (26.42% relative abundance) and ^{13}CL (0.68% relative abundance) fractions and a 23-fold enrichment between the ^{13}CH and the ^{12}CH control fractions (1.14%). In terms of fold change, however, *Pseudomonas* was the most enriched genus with a 26-fold increase in abundance between ^{13}CH (15.64% relative abundance) and ^{13}CL (0.59% relative abundance) fractions of $^{13}CO_2$ incubated plants (Fig 2). 15 out of the 28 genera were found to be metabolising exudates in the endosphere but not the rhizosphere, whereas 16 out of 29 genera were metabolising exudates in the rhizosphere but not the endosphere. The remaining 13 genera were enriched in the heavy fractions of both the endophytic and rhizospheric compartments, suggesting that they are able to survive and make use of plant metabolites in both of these niches (Table S5).

Figure 2. Eight genera of bacteria isolated from the endophytic compartment of *Arabidopsis thaliana* plants whose relative abundance demonstrated the greatest overall enrichment between the ^{13}CH fraction and the ^{12}CH fraction due to the metabolism of ^{13}C labelled root exudates. In comparison, the genus *Streptomyces* did not demonstrate enrichment between these fractions suggesting they did not metabolise labelled root exudates. 12CL/13CL and 12CH/13CH are the buoyant density fractions representing the light/unlabelled (L) and

heavy/labelled (H) fractions of DNA from $^{12}CO_2$ (12C) or $^{13}CO_2$ (13C) incubated plants respectively.



Surprisingly, there was no enrichment of *Streptomyces* bacteria in the ^{13}CH fractions of either the rhizosphere or endosphere, despite being the most dominant member of the phylum Actinobacteria in both compartments (Figs 1, 2 and S3). Taken together, these data suggest that, in soil, *Streptomyces* bacteria were outcompeted for the root exudates by unicellular bacteria, particularly Proteobacteria, under the conditions used in this experiment. In the current study, Proteobacteria were very abundant in the unfractionated soil (35% average relative abundance), rhizosphere (39%) and endophytic compartment (91%), compared to Actinobacteria (present at 20%, 15% and 3% in the soil, rhizosphere and root compartments, respectively) (Fig S1) and were therefore likely to have the upper hand at the outset of competition (Scheuring and Yu 2012). Accordingly, genera that were found to be metabolising the greatest amount of exudates in the roots were also found to be enriched in the endophytic compartment compared to the surrounding soil (Fig S4). Given their diverse metabolic capabilities, it is likely that streptomycetes are able to persist at low abundance by feeding on more complex organic polymers that contained a much lower level of labelled ^{13}C, under conditions of high competition or in the absence of a preferred type of exudate. *Arabidopsis*

thaliana has also been shown to have a relatively small "rhizosphere effect" compared to other plant species (Bulgarelli et al 2015, Schlaeppi et al 2014), with weak differentiation in terms of microenvironment and community composition between the rhizosphere and bulk soil. Therefore, complex polymers may have been more readily available in the rhizosphere than exudates, particularly given the fact that compost (used as the plant growth medium in this experiment) contains a high level of organic matter. Alternatively, as they also have spore-forming capabilities, it is possible that *Streptomyces* species were mostly dormant in the rhizosphere and endosphere when the plants were being pulse-labelled with $^{13}CO_2$.

Isolation and genome sequencing of *Streptomyces* endophyte strains. To analyse plant root interactions with *Streptomyces* species, we selectively isolated these bacteria from *A. thaliana* roots and generated high-quality genome sequences for five phylogenetically distinct *Streptomyces* strains, as judged by initial 16S rRNA gene amplification and sequencing. We also generated high-quality genome sequences for three known endophyte strains (Table 1). These are *Streptomyces lydicus* isolated from the commercial plant growth-promoting product Actinovate and two additional *S. lydicus* strains (ATCC25470 and ATCC31975) obtained from the American Type Culture Collection (Tokala et al 2002). We submitted the genome sequences of all nine strains to the antiSMASH 4.0 server which predicts secondary metabolite biosynthetic gene clusters (Weber et al 2015). The results predicted that all nine strains encode siderophores, antifungal and antibacterial natural products, all of which are common in this genus. To test their bioactivities, agar plate bioassays were performed and showed that all the *Streptomyces* strains inhibited the growth of at least one pathogenic microorganism while some, such as strain N2, show broad spectrum antifungal and antibacterial activity, including against the Gram-negative bacterium, *E. coli* (Table S4).

Table 1. Features of the *Streptomyces* endophyte genomes sequenced for this study. Biosynthetic gene clusters (BGCs) were predicted using antiSMASH 4.0.

Strain	Accession no.	Genome size (bp)	ORFs	tRNAs	rRNAs	BGCs
L2	QBDT00000000	8,073,926	7079	68	18	32
M2	CP028834	8,718,751	8026	72	18	24

M3	QANR00000000	8,304,843	7561	74	18	28
N1	QBDS00000000	7,207,104	6239	65	21	22
N2	CP028719	8,428,700	7401	69	21	35
Actinovate	RDTC00000000	9,139,876	7989	67	21	36
ATCC25470	RDTD00000000	7,935,716	7084	65	21	26
ATCC31975	RDTE00000000	9,244,118	8128	66	21	34

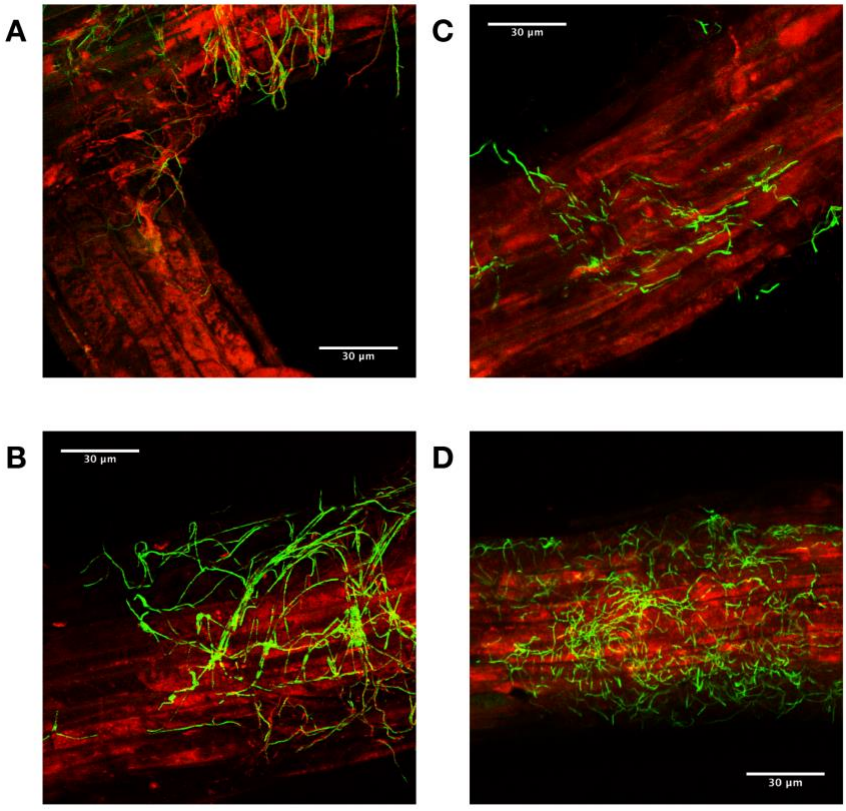
Some plant growth-promoting bacteria are capable of producing plant phytohormones and KEGG pathway analysis revealed that the genomes of all our sequenced *Streptomyces* strains possess genes encoding proteins involved in the biosynthesis of indole-3-acetic acid (IAA), which can contribute to shoot and root growth (Remans et al 2006) (Table S6). For example, all strains have genes encoding key proteins involved in the indole-3-acetamide (IAM) pathway, whereby tryptophan is converted to IAM via a tryptophan 2-monooxygenase enzyme (KEGG reaction R00679). IAM is then further converted to IAA through the action of an amidase enzyme (KEGG reaction R03096). Several strains also possessed genes encoding enzymes involved in the tryptamine (TAM) pathway, which converts tryptamine to IAA via an amine oxidase enzyme (R02173) and an acetaldehyde oxidase enzyme (R02681). *In vitro* colorimetric assays using Salkowski's reagent (Bric et al 1991) qualitatively confirmed the ability of all strains to make IAA (Fig. S5).

In addition to IAA, the genomes of all streptomycete isolates possess up to two copies of genes encoding the enzyme aminocyclopropane-1-carboxylate (ACC) deaminase. This cleaves ACC, which is the direct precursor for the plant phytohormone ethylene, into ammonia and 2-oxobutanoate, Kegg reaction: R00997 (Table S6). Bacteria can use the products of this reaction as a nitrogen source, and it was demonstrated *in vitro* that all of the isolates were capable of utilising ACC as a sole nitrogen source when added to minimal medium (Fig. S6). There is evidence that the activity of this enzyme can reduce damage and early-onset senescence caused by excessive ethylene production under prolonged periods of plant stress, by removing the substrate for ethylene biosynthesis (Glick 2014, Palaniyandi et al 2014, Yang et al 2009).

Not all *Streptomyces* strains have growth promoting effects in *A. thaliana.* To investigate whether the sequenced *Streptomyces* strains promote plant growth and fitness, we established root infection assays in which seeds were coated with a suspension of pre-germinated

Streptomyces spores - these were also added to the soil growth substrate. Tagging the strains with eGFP and the apramycin resistance (*aac*) gene allowed visual confirmation of root infection using confocal microscopy (Fig. 3) and selective re-isolation of the strains on agar plates containing apramycin.

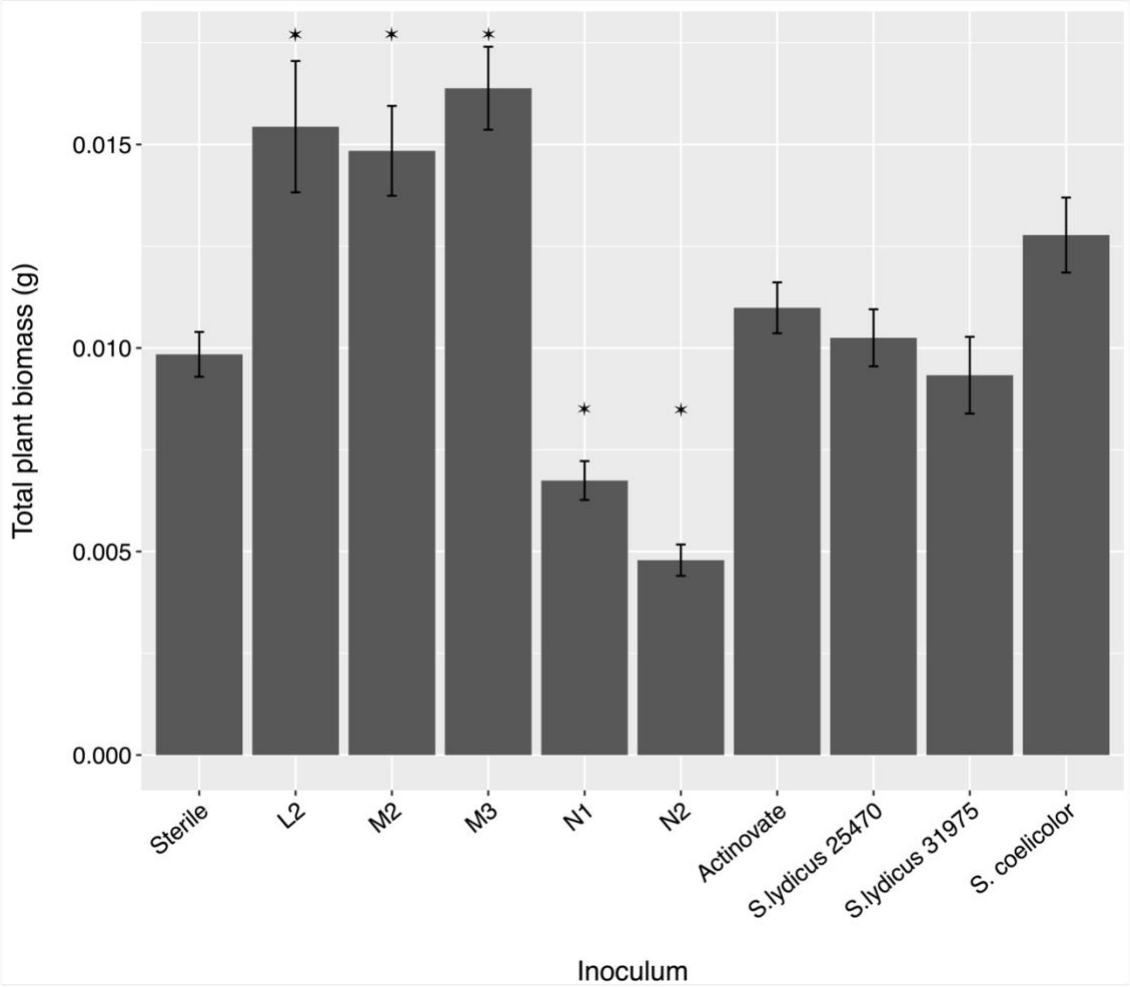
Figure 3. Confocal Laser Scanning Microscopy images of *Arabidopsis thaliana* rhizoplane colonisation by eGFP-tagged *Streptomyces* strains three days after inoculation. A and B show *A. thaliana* roots (red) colonised by eGFP-tagged *Streptomyces coelicolor* M145 (green) and images C and D show *A. thaliana* roots (red) colonised by eGFP-tagged *Streptomyces* endophyte strain M3 (green).



We tested all the genome-sequenced stains from this study alongside the model organism *S. coelicolor* M145. All strains were able to colonise plant roots with varying efficiencies, and several of them significantly increased plant biomass *in vitro* (Fig. 4). However, not all of them promoted plant growth; application of *Streptomyces* strains N1 and N2 significantly reduced the growth of *A. thaliana* ($P < 0.05$ in Tukey's HSD, Fig. 4) and in some cases led to a senescence phenotype with leaf browning; it is possible that some of the biosynthetic gene

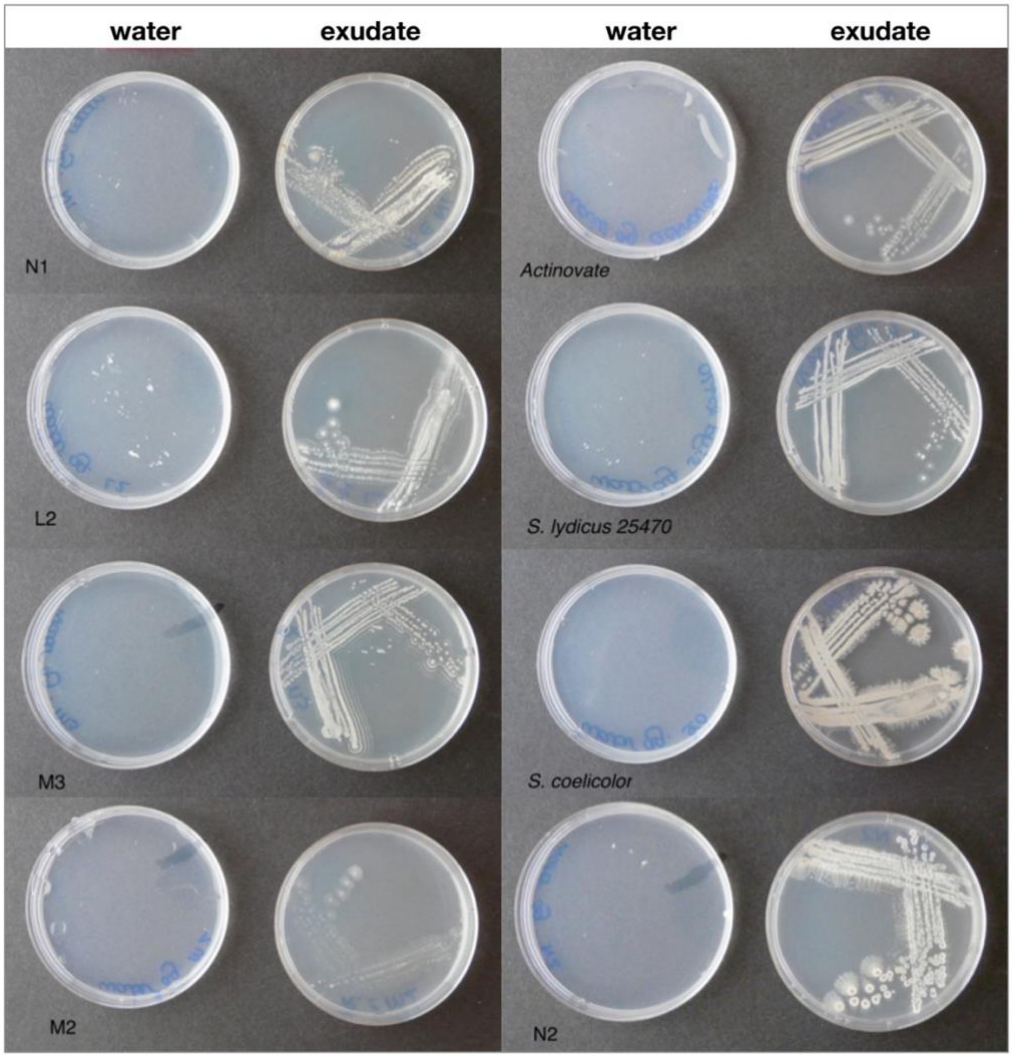
clusters which have a low percentage homology to other known secondary metabolite clusters, may encode novel herbicidal compounds in these strains.

Figure 4. The impact of *Streptomyces* isolates on *A. thaliana* biomass (grams) 16 days after inoculation. * represents a significant difference in biomass relative to sterile control plants ($P < 0.05$).



Root exudates promote the growth of *Streptomyces* endophytes *in vitro* but not *in vivo*. Plants are known to use their root exudates to recruit bacteria from the soil, and these exudates include a complex mixture of sugars, amino acids and phytohormones, including jasmonic acid (JA) and salicylic acid (SA). However, our DNA-SIP experiments suggested that *Streptomyces* species were not feeding on these root exudates in soil-grown plants (Figs 2 and S3). To test whether streptomycetes could use root exudates when grown in monoculture, we collected exudates from hydroponically-grown wild-type *A. thaliana* plants and grew *Streptomyces* isolates on agarose plates containing water only, or water plus sterile root exudates. None of

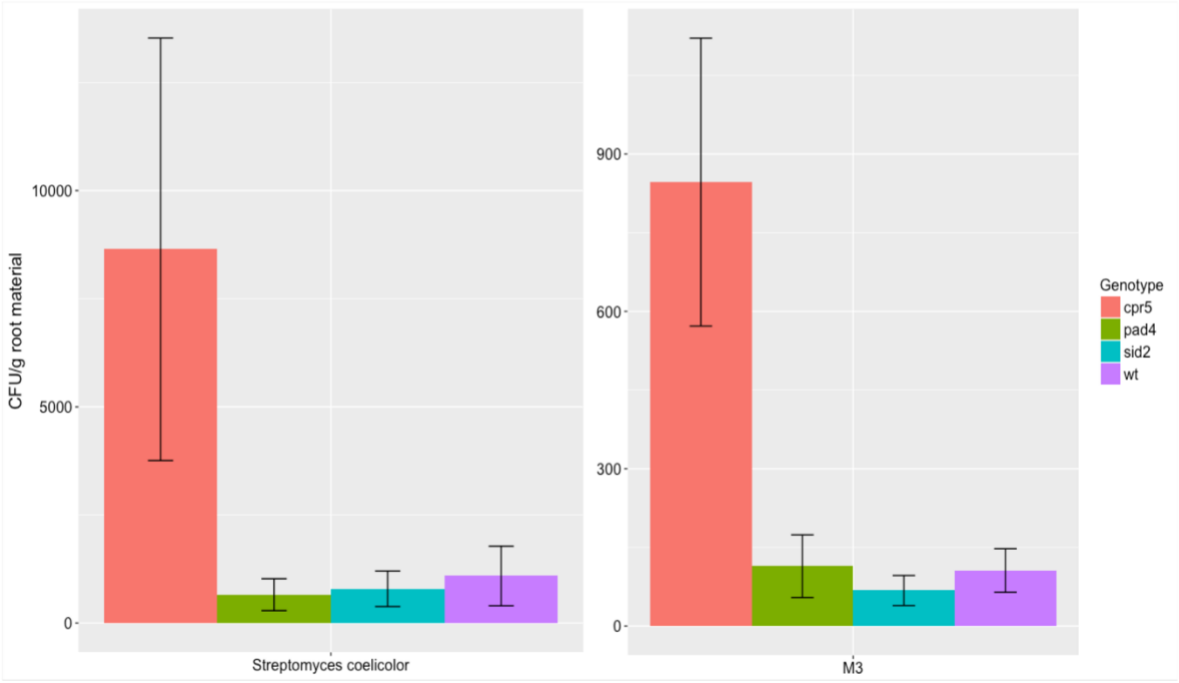
519 the strains grew on agarose alone, but all grew on agarose plates containing root exudates (Fig.
520 5).
521
522 **Figure 5.** *Streptomyces* strains grown on agarose dissolved in sterile water or agarose dissolved
523 in filter sterilised *A. thaliana* root exudates. Strains M2, M3, N1, N2, and L2 are the
524 *Streptomyces* endophytes isolated and genome sequenced in this study. The other strains are *S.*
525 *lydicus* isolated from Actinovate, *S. lydicus* ATCC25470 and the model laboratory strain *S.*
526 *coelicolor* M145.



527
528 **Streptomycetes do not feed on salicylic acid.** Previous studies have suggested that the
529 defence phytohormone SA plays a key role in modulating root microbiome assembly in *A.*
530 *thaliana*, particularly in recruiting streptomycetes, and that a *Streptomyces* endophyte strain
531 isolated from *A. thaliana* roots could grow using SA as a sole carbon source (Lebeis et al 2015).
532 To test this, we first compared root colonisation efficiency by *S. coelicolor* M145 and
533 *Streptomyces* M3 in wild-type *A. thaliana* col-0, *cpr5* mutant plants that constitutively produce

SA, and in *pad4* and *sid2-2* plants that are deficient in SA production (Bowling et al 1994, Jirage et al 1999, Wildermuth et al 2001, Zhou et al 1998). The results confirmed that root colonisation (measured by counting CFU returned per gram of plant root) was significantly affected by plant genotype, irrespective of the *Streptomyces* strain used as an inoculum (plant genotype on log-transformed CFU g^{-1} was P <0.01, strain genotype interaction was P = 0.67 in an anova test). Specifically, the colonisation of both M145 and M3 was significantly increased in *cpr5* mutant plants (Fig. 6), which constitutively make SA, compared to the other three genotypes ($P < 0.05$ in all Tukey's HSD tests between *cpr5* and other plant genotypes).

Figure 6. Increased root colonisation of *Arabidopsis thaliana cpr5* plants (constitutively expressing salicylic acid) by *Streptomyces* strains compared to *pad4* and *sid2-2* plants (deficient in salicylic acid production) and wild-type Col-0 plants. Root colonisation was measured as the average colony forming units (±SE) of *Streptomyces*, per gram of root, that could be re-isolated from plants 4 weeks after germination**.** N = 6 plants per treatment.



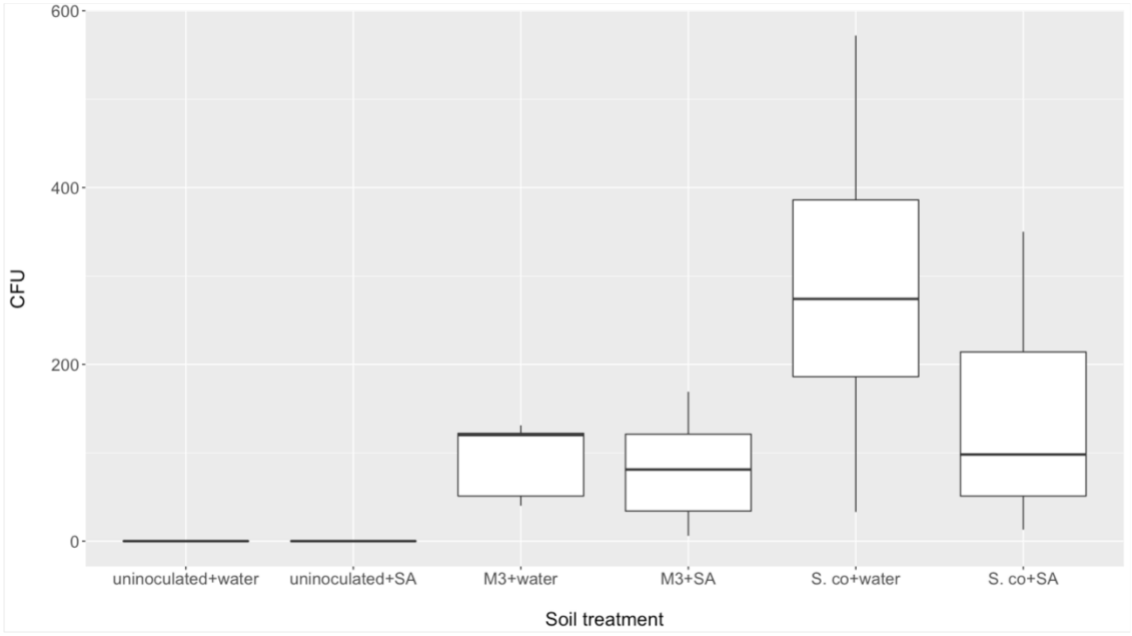
However, we observed no significant difference between the wild-type, *pad4*, or *sid2-2* plants (Fig. 6). We note that the *cpr5* gene (deleted in *cpr5* plants) has a complex role in regulating plant growth, immunity, and senescence (Jing et al 2007). The *cpr5* plants demonstrated weak growth compared to the other genotypes (Fig. S7), giving rise to the possibility that the observed increase in streptomycete colonisation was due to the complex phenotype of the *cpr5*

554 mutant plants and not necessarily the higher levels of SA produced by these plants (Jing et al
555 2008).

556 In order to test whether SA could be directly used as a chemoattractant by *Streptomyces*
557 species, we grew all of the sequenced streptomycete strains next to paper disks soaked in either
558 0.5 mM SA, 1 mM SA, or 0.1% (v/v) DMSO (a control for the solvent) but observed no obvious
559 growth towards SA after 10 days. (Fig. S8). We then reasoned that altered exudate SA levels
560 might indirectly benefit streptomycetes by negatively modulating the levels of the other
561 rhizobacteria in the soil. We tested this by establishing soil microcosms in deep 12 well plates,
562 which were then wetted with either sterile water or 0.5 mM SA. Microcosms were inoculated
563 with spores of M145, M3, or left as uninoculated controls. Nine replicates of each wetting
564 treatment were run in parallel for each strain and the control. Strains were recovered on
565 apramycin-containing selective agar medium after 10 days. A GLM with a negative binomial
566 distribution was used to model the effect of strain (M145 or M3), soil treatment (SA or water),
567 and their interaction on bacterial CFU returned from soil wells. Although there was overall a
568 significantly greater number of CFU of M145 recovered from soil wells than for the M3 strain
569 ($P < 0.01$), there was no significant effect of soil treatment ($P = 0.67$) on CFU number. The
570 interaction term was also insignificant ($P = 0.20$), indicating that this did not differ between
571 the two inoculated strains (Fig. 7). This suggested that neither strain had a competitive
572 advantage when greater concentrations of SA were present.

573 The simplest explanation of these results is that SA does not specifically attract
574 streptomycetes to the rhizosphere of *A. thaliana*. This does not necessarily contradict the
575 previously published observation (in Lebeis et al 2015) that SA is being used as a carbon source
576 by streptomycetes, as this has been demonstrated before in another streptomycete isolate
577 (Ishiyama et al 2004). However, we observe that the supplementary methods of this paper state
578 that the minimal medium agar with 0.5 mM SA that was used to grow *Streptomyces* sp. 303
579 also contained 1 g L^{-1} (3.875 mM) sodium citrate, which is an additional candidate source of
580 carbon and energy. To test this, we grew all the genome-sequenced strains from our study on
581 minimal medium (MM) agar and MM agar containing 0.5mM SA, 3.875 mM sodium citrate,
582 or their preferred carbon source (either 5 gL^{-1} mannitol, maltose or sucrose). All the strains
583 grew well on their preferred carbon source and most strains also grew on sodium-citrate-
584 containing plates, but we observed no growth on MM agar with or without SA (Fig. S9). Our
585 results suggest that SA is not used as a carbon source by the bacteria used in our study, and this
586 is additionally supported by the absence of known SA degradation genes in the genomes of all
587 the sequenced isolates (Table S3).

Figure 7. Number of colony forming units (CFU) of inoculated strains returned from soil microcosms treated with either SA or dH_2O after 10 days. Microcosms were inoculated with spores of either *S. coelicolor* (S.co), M3 or left uninoculated as a control. N = 9 microcosms per treatment.



Taking these results together, we conclude that the observed increase in colonisation by streptomycetes in *cpr5* mutant plants was likely due to a pleiotropic effect of plant genotype rather than the presence of SA. This is consistent with the observation that there was no effect of attenuated SA production in the *pad4* and *sid2-2* plants on colonisation, and the observation that *cpr5* plants have been noted to undergo early senescence and overproduce reactive oxygen species (Jing et al 2007, Jing et al 2008, Kirik et al 2001). Overall, we found no evidence to support the hypothesis that SA specifically attracts streptomycetes to *A. thaliana* roots, but rather they may have better access to the roots of senescing *cpr5* plants due to their saprotrophic and filamentous lifestyle.

Discussion.

Here we used DNA-SIP to identify the bacteria feeding on exudates in the *A. thaliana* rhizosphere and endosphere. To our knowledge, this is the first report of DNA-SIP on the endosphere compartment of *A. thaliana*. The role of Proteobacteria and Firmicutes have previously been investigated in the rhizosphere, however this was using DNA-SIP combined with a Denaturing Gradient Gel Electrophoresis approach instead of with 16S rRNA amplicon

sequencing (Haichar et al 2012). Our results, combined with previous published reports on the chemistry of *A. thaliana* exudates, suggest that root exudates are a rich and public food resource (Badri and Vivanco 2009, Bais et al 2006, Haichar et al 2012). That is, many different genera of bacteria appear to be able to use root exudates as a carbon source, as they contain a diverse range of widely used metabolites including sugars, amino acids, and phenolic compounds (Badri and Vivanco 2009, Chaparro et al 2013). In our study, bacteria that could metabolise root exudates were those that have previously been noted for their positive influence on plant fitness and were enriched in the endophytic compartment. For example, members of the genus *Pseudomonas* are known to produce many bioactive and growth-promoting molecules (Glick et al 1997, Hernández-León et al 2015, Mercado-Blanco et al 2016, Raza et al 2016), and *Rhizobia* have been shown to have a positive effect on root development and plant biomass in *Arabidopsis thaliana* (Zhao et al 2017). We hypothesise that this apparent mutualism between plant and bacteria is evolutionarily stabilised via a Partner Fidelity Feedback mechanism (Foster and Wenseleers 2006, Weyl et al 2010). Bacterial colonies that release growth-promoting factors and/or antibiotics exploit a widespread pre-adaptation in plants to proliferate root biomass in nutrient-rich or disease-free soils. In return, plant root masses release food resources for bacteria which promote the local colony growth. Root-derived resources are also likely to fuel interference competition amongst microbes, with the priority advantage given to the most abundant strains. In our SIP experiment, these were the proteobacterial genera (Scheuring and Yu 2012). Interference competition amongst bacteria typically manifests as competitive exclusion by taking up niche space, plus the secretion of antibacterials and antifungals, the latter of which may indirectly benefit the plant by inhibiting the growth of pathogens.

However, such interactions can be dynamic over time since the composition of plant root exudates is known to fluctuate considerably over the plant life cycle. The abundance of particular microbial taxa has been shown to correlate with these changes, suggesting some specificity in exudate usage (Chaparro et al 2013, Chaparro et al 2014, Haichar et al 2012, Zhalnina et al 2018). Interestingly streptomycetes, the main focus of our study, did not feed off root exudates in our SIP experiment despite being able to grow on purified root exudates *in vitro*. *Streptomyces* were still present at low abundance in the endosphere and rhizosphere, suggesting that their preferred substrates might have been absent from root exudates at the time of ^{13}C labelling, or that they were largely out-competed by the more abundant proteobacterial genera. *Streptomyces* are spore-formers and thus may enter a dormant phase in their lifecycle under unfavourable conditions (van der Meij et al 2017). As such, they may have been

644 detectable via their DNA, but dormant, under conditions in the root and rhizosphere at the time
645 of sampling. It should also be noted that *Streptomyces* are effective saprophytes, and so it is
646 possible that they feed off older, plant-originated organic material that had been sloughed off
647 before the ^{13}C labelling SIP experiment or on similar matter in the compost growth medium.

648 Our study shows that *Streptomyces* strains isolated from *A. thaliana* plant roots encode
649 an array of plant beneficial traits, including those involved in plant-growth promotion and
650 protection against infection. If such strains are to be used successfully as biocontrol and
651 growth-promoting agents, the next challenge is to determine how to ensure consistent
652 establishment in the rhizosphere and endosphere and how to promote the activity of these
653 strains within the plant root microbiome. Previous studies have suggested that phenolic
654 compounds, particularly SA, can directly affect the abundance of streptomycetes in the root
655 microbiome (Lebeis et al 2015). However, such studies have made use of *Arabidopsis* lines,
656 such as *cpr5* plants, that have mutations in key regulatory pathways. We suggest that such
657 mutations result in pleiotropic effects that make it difficult to determine causal relationships
658 between any given compound and bacterial colonisation. Thus, a more targeted approach is
659 required to determine if particular exudates, in addition to the initial abundance of strains in
660 the starting soil inoculum, are important for recruiting a higher abundance of *Streptomyces*
661 species to plant roots.

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669 **Conflicts of interest.** The authors declare no conflicts of interest.

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