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Investigating the Role of Root Exudates in Recruiting *Streptomyces* Bacteria to the *Arabidopsis thaliana* Microbiome

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Streptomyces species are saprophytic soil bacteria that produce a diverse array of specialized metabolites, including half of all known antibiotics. They are also rhizobacteria and plant endophytes that can promote plant growth and protect against disease. Several studies have shown that streptomycetes are enriched in the rhizosphere and endosphere of the model plant *Arabidopsis thaliana*. Here, we set out to test the hypothesis that they are attracted to plant roots by root exudates, and specifically by the plant phytohormone salicylate, which they might use as a nutrient source. We confirmed a previously published report that salicylate over-producing *cpr5* plants are colonized more readily by streptomycetes but found that salicylate-deficient *sid2-2* and *pad4* plants had the same levels of root colonization by *Streptomyces* bacteria as the wild-type plants. We then tested eight genome sequenced *Streptomyces* endophyte strains *in vitro* and found that none were attracted to or could grow on salicylate as a sole carbon source. We next used $^{13}CO_2$ DNA stable isotope probing to test whether *Streptomyces* species can feed off a wider range of plant metabolites but found that *Streptomyces* bacteria were outcompeted by faster growing proteobacteria and did not incorporate photosynthetically fixed carbon into their DNA. We conclude that, given their saprotrophic nature and under conditions of high competition, streptomycetes most likely feed on more complex organic material shed by growing plant roots. Understanding the factors that impact the competitiveness of strains in the plant root microbiome could have consequences for the effective application of biocontrol strains.

Keywords: ***Streptomyces*, arabidopsis, root exudates, endophytes, rhizosphere**

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INTRODUCTION

Streptomyces species are saprophytic soil bacteria that play an important ecological role as composters in soil and are prolific producers of antimicrobial compounds (van der Meij et al., 2017; Hutchings et al., 2019). The genus first evolved ca. 440 million years ago shortly after plants began to colonize land and it has been suggested that their filamentous growth and diverse secondary metabolism may have evolved to enhance their ability to colonize plant roots (Chater, 2016). Several studies have reported that streptomycetes are abundant inside the roots of the model plant

Arabidopsis thaliana (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014; Bai et al., 2015; Carvalhais et al., 2015) where they can have beneficial effects on growth (Worsley et al., 2020) while 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 pathogenic infection (Viaene et al., 2016; Newitt et al., 2019). However, different plant genotypes are associated with distinctive root-associated microbial communities and not all plant species enrich streptomycetes in their roots, a notable example being barley (Bulgarelli et al., 2015). This suggests that either specific selection mechanisms exist that enable different plant hosts to recruit particular microbial species from the soil and/or that bacterial taxa such as *Streptomyces* species colonize some plants better than others.

In our previous work, we reported that three *Streptomyces* strains isolated from the roots of *A. thaliana* plants can be re-introduced to new plants to promote their growth both *in vitro* and in soil. Furthermore, one of these strains exhibited broad spectrum antifungal activity and, when its spores were used as a seed coating, it colonized the roots of bread wheat plants and protected them against the commercially important pathogen *Gaeumannomyces tritici*, the causative agent of take-all disease (Worsley et al., 2020). Here we set out to test the hypothesis that streptomycetes are attracted to, and metabolize, components of the *A. thaliana* root exudate. Plants are known to release up to 40% of their photosynthetically fixed carbon into the surrounding soil via their roots and bacterial species in the soil have been shown to be attracted to, and be capable of metabolizing, specific resources contained within these root exudates (Haichar et al., 2012; Badri et al., 2013; Haichar et al., 2016). In turn, it is thought that the plant host might establish a beneficial root microbiome by producing particular types of root exudates that attract microbial species with desirable metabolic traits (Bais et al., 2006; Badri and Vivanco, 2009). Recent studies have further suggested that defence phytohormones, which are accumulated by plants in response to pathogenic attack, may play a key role in modulating the establishment of the normal *A. thaliana* root microbiome, since mutant plants that are disrupted in these pathways accumulate significantly different microbial communities (Carvalhais et al., 2013; Carvalhais et al., 2015; Lebeis et al., 2015; Liu et al., 2017). In particular, the abundance of bacteria in the *A. thaliana* rhizosphere and roots has been shown to correlate with concentrations of the defence phytohormone salicylic acid (Lebeis et al., 2015). Plant growth hormones such as indole-3-acetic acid have also been shown to modulate the production of *Streptomyces* specialized metabolites which may aid their competitive establishment in the root microbiome (Worsley et al., 2020).

Here we set out to test the hypothesis that streptomycetes are attracted by, and feed on, salicylate as well as other components of *A. thaliana* root exudates. We used genome sequenced *Streptomyces* endophyte strains that we isolated in a previous study (Worsley et al., 2020), including three which have been shown to promote *A. thaliana* growth *in vitro* and in compost, and assessed their ability to colonize mutant *A. thaliana* plants with altered salicylate production. Our results indicate that salicylate does not attract or feed these *Streptomyces* species, at least under the conditions used in our experiments. Furthermore, $^{13}CO_2$ DNA stable isotope probing (DNA-SIP) of wild-type *A. thaliana* plants grown in compost show that *Streptomyces* bacteria do not feed on root exudates and are instead outcompeted by faster growing proteobacteria. We propose that streptomycetes are more likely to feed on complex organic matter that is not labeled in the $^{13}CO_2$ DNA-SIP experiments. This work is an important step in understanding the role of these bacteria in the plant rhizosphere and also reveals which members of the *A. thaliana* microbiome feed on plant metabolites.

RESULTS

Root colonization by *Streptomyces* bacteria is affected by plant genotype. A previous study reported that *A. thaliana cpr5* plants that constitutively produce salicylate have an altered root microbiota compared to wild-type plants, and that certain streptomycetes were better able to colonize these plants *in vitro*, due to their ability to metabolize salicylate (Lebeis et al., 2015). This suggests that salicylate might be directly responsible for recruiting these bacteria to the plant roots. To test this hypothesis, we compared root colonization efficiencies by *S. coelicolor* M145 and the root-associated strain *Streptomyces* M3 in wild-type *A. thaliana* Col-0 plants, *cpr5* plants as well as two other genotypes, *pad4* and *sid2-2* plants, that are deficient in salicylate production (**Table 1**) (Zhou et al., 1998; Jirage et al., 1999; Wildermuth et al., 2001; Jing et al., 2007). To measure plant root colonization efficiency, we established root infection assays in which pre-germinated *Streptomyces* spores were used to coat *A. thaliana* seeds and were also added to the surrounding soil. *Streptomyces* strains M3 and *S. coelicolor* M145 (**Table 1**), were used because they have previously been observed to interact extensively with the *A. thaliana* roots using confocal microscopy (Worsley et al., 2020). Both strains have been engineered to carry the apramycin resistance (*aac*) gene, enabling the selective re-isolation of these strains from the roots of soil grown plants and their subsequent enumeration on agar plates containing apramycin. Colonization was measured as colony forming units (cfu) retrieved per gram of plant root tissue. This method has been used previously to assess colonization efficiency by other streptomycete strains in the plant root microbiome (Bonaldi et al., 2015; Chen et al., 2016). The results of these assays show that root colonization was significantly affected by plant genotype (**Figure 1**), irrespective of the *Streptomyces* strain used as an inoculum; plant genotype had a significant effect on the log-transformed cfu g^{-1} ($F_{(3,39)} = 6.17$, $p < 0.01$), whereas the strain-genotype interaction term was insignificant ($F_{(3,39)} = 0.51$, $p = 0.68$) in an ANOVA test. Indeed, consistent with previous findings (Lebeis et al., 2015), colonization by both strains significantly increased in the *cpr5* mutant plants which constitutively make salicylate (**Figure 1**), compared to wild-type plants and the salicylate-deficient plants, *pad4* and *sid2-2* ($p < 0.05$ in all Tukey's HSD tests between *cpr5* and the other plant genotypes). However, we

TABLE 1 | Bacterial strains and plants used in experiments.

Strain name	Description	Origin	Accession number	References
Streptomyces L2	Wild-type	*A. thaliana* roots	QBDT00000000	Worsley et al. (2020)
Streptomyces M2	Wild-type	*A. thaliana* roots	CP028834	Worsley et al. (2020)
Streptomyces M3	Wild-type	*A. thaliana* roots	QANR00000000	Worsley et al. (2020)
Streptomyces N1	Wild-type	*A. thaliana* roots	QBDS00000000	Worsley et al. (2020)
Streptomyces N2	Wild-type	*A. thaliana* roots	CP028719	Worsley et al. (2020)
Streptomyces lydicus ATCC25470	Wild-type	American Type Culture Collection	RDTD00000000	Worsley et al. (2020)
Streptomyces coelicolor M145	Wild-type	John Innes Centre	AL645882	Bentley et al. (2002)
Streptomyces lydicus Actinovate	Wild-type	Isolated from Actinovate™	RDTC00000000	Worsley et al. (2020)
Streptomyces M3-eGFP	*Streptomyces* M3 containing the plasmid pIJ8660/*ermEp*-egfp* (**Supplementary Table S2**)	This study	–	–
Streptomyces coelicolor M145-eGFP	*Streptomyces coelicolor* M145 containing the plasmid pIJ8660/*ermEp*-egfp* (**Supplementary Table S2**)	This study	–	–
Arabidopsis thaliana Col-0	Wild-type, ecotype Col-0	UEA lab stock	–	Worsley et al. (2020)
Arabidopsis thaliana cpr5	*cpr5-2* genotype in Col-0 genetic background, gene code At5g64930, constitutive expression of PR genes and high levels of salicylate	Nottingham *Arabidopsis* Stock Centre (NASC)	–	Kirik et al. (2001)
Arabidopsis thaliana sid2-2	*sid2-2* genotype in a Col-0 genetic background, gene code At1g74710, deficient in induction of salicylate accumulations	NASC	–	Wildermuth et al. (2001)
Arabidopsis thaliana pad4	*pad4-1* genotype in a Col-0 genetic background, gene code At3g52430, phytoalexin deficient and deficient in salicylate accumulation	NASC	–	Jirage et al. (1999)

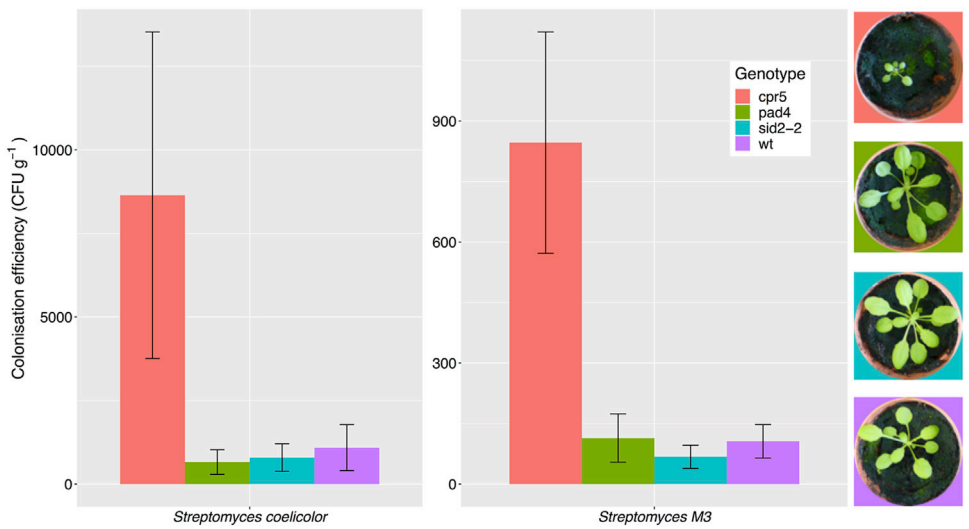

FIGURE 1 | *Streptomyces coelicolor* M145 and the *A. thaliana* root endophyte strain *Streptomyces* M3 show increased root colonization of *Arabidopsis thaliana cpr5* plants, which constitutively produce salicylic acid, compared to *pad4* and *sid2-2* plants, which are deficient in salicylic acid production, and wild-type (wt) Col-0 plants. Root colonization was measured as the average colony forming units (±SE) of *Streptomyces* bacteria per gram of root, that could be re-isolated from plants four weeks after germination. *N* = 6 plants per treatment. Plant growth phenotypes are shown for comparison.

observed no significant difference in root colonization by M145 or M3 in *pad4* or *sid2-2* plants, compared to wild-type *A. thaliana* plants ($p > 0.05$ in Tukey's HSD tests), suggesting that reduced quantities of salicylate do not, in turn, correspond to a reduction in colonization by streptomycetes (**Figure 1**). We note that the *cpr5* gene has a complex role in regulating pathways involved in plant growth, immunity, and senescence (Jing et al., 2007) and that the *cpr5* plants grown in our experiments were compromised in their growth compared to all the other plant genotypes tested here (**Figure 1**). The altered development of *cpr5* plants has been observed in numerous other studies (Kirik et al., 2001; Jing et al., 2007; Brininstool et al., 2008; Jing et al., 2008; Doornbos et al., 2011; Gao et al., 2011) and gives rise to the possibility that the observed increase in colonization by *Streptomyces* strains M145 and M3 was linked to other aspects of the complex phenotype of the *cpr5* plants and was not necessarily due to the higher levels of salicylate produced by these plants.

Streptomyces Species are Not Attracted by and Do Not Feed on Salicylic Acid *In Vitro*

To test whether the streptomycete strains used in recolonization experiments can utilize salicylate as a sole-carbon source, we grew each strain on minimal agarose medium (MM) containing either sodium citrate, or their preferred carbon sources (mannitol, maltose or sucrose). We also tested seven additional strains, four of which were also isolated from *A. thaliana* roots, as well as three strains of *Streptomyces lydicus* which are known colonizers of plant roots and were genome-sequenced in a previous study (Worsley et al., 2020). All the strains grew well on their preferred carbon source but none of the strains grew on MM plates containing salicylate as the sole carbon source (**Supplementary Figure S1**). This included *Streptomyces* M3 which had demonstrated increased levels of colonization in *A. thaliana cpr5* plants (**Figure 1**). Thus, salicylate is not used as a carbon source by any of the *Streptomyces* strains used in our study, and this is supported by the absence of known SA degradation genes in the genomes of all these isolates (**Supplementary Table S1**). Rather than acting as a nutrient source, some root exudate molecules may recruit bacteria to the root niche by acting as chemoattractants (Rudrappa et al., 2008). In order to test whether salicylate is a chemoattractant for *Streptomyces* species, we grew our test strains on agar plates next to paper disks soaked in either 0.5 mM or 1 mM salicylate, or 0.1% (v/v) DMSO (solvent control) but observed no growth toward salicylate after 10 days (**Supplementary Figure S2**).

To test whether altered salicylate levels might indirectly benefit streptomycetes by negatively modulating the levels of the other rhizobacteria in the soil and removing competition, we established soil microcosms in deep 12-well plates, which were wetted with either sterile water or 0.5 mM salicylate. Microcosms were inoculated with spores of *S. coelicolor* M145-eGFP or *Streptomyces* M3-eGFP, both of which had colonized *cpr5* plants more effectively than wild-type plants. Nine replicates of each treatment were run in parallel for each strain and the uninoculated control. Strains were recovered on apramycin-containing selective agar medium after 10 days. No apramycin resistant *Streptomyces* colonies were re-isolated from the control soil wells, indicating that any resulting colonies arising from the treated wells were derived from inoculated strains (**Figure 2**). A generalized linear model (GLM) with a negative binomial distribution demonstrated that, overall, a significantly greater number of *S. coelicolor* M145-eGFP colonies could be recovered from soil wells than *Streptomyces* M3-eGFP ($p < 0.002$; **Figure 2**). However, there was no significant effect of the different soil wetting treatments (either water or salicylate) on CFU number ($p = 0.07$; **Figure 2**). The interaction term between strain and wetting treatment was also insignificant ($p = 0.20$), indicating that this did not differ between the two inoculated strains. These results suggest that neither *Streptomyces* strain had a competitive advantage when greater concentrations of SA were present in soil. Taking these results together, we conclude that the observed increase in colonization by strains M145 and M3 in *A. thaliana cpr5* plants is likely due to a pleiotropic effect of plant genotype on other aspects of plant growth rather than a direct effect of the increased presence of SA in root exudates and soil.

Streptomyces Species Feed on Root Exudates *In Vitro* but not in Soil

We then set out to test the hypothesis that *Streptomyces* bacteria can grow using a wider range of *A. thaliana* root exudate molecules as their sole nutrient source. We tested two *S. lydicus* strains, the five *Streptomyces* strains isolated from surface-washed *A. thaliana* roots (Worsley et al., 2020) and the model organism *S. coelicolor* M145 (**Table 1**). *A. thaliana* root exudates were collected using a small-scale hydroponics system and used to make plates with purified agarose. Agarose was used rather than agar, because streptomycetes can grow on the impurities in agar (not shown). Control plates were made using agarose and sterile water. The results show that all eight strains could grow on plates containing *A. thaliana* root exudates but not on the water-only control plates (**Supplementary Figure S3**) implying that all of the *Streptomyces* strains tested here can use *A. thaliana* root exudate as their sole food source *in vitro*.

Several independent studies have reported that *A. thaliana* plants have a relatively stable and consistent root bacterial community, which is enriched with members of the phylum Actinobacteria; this enrichment is predominantly driven by the presence of the family *Streptomycetaceae* (Bulgarelli et al., 2012; Lundberg et al., 2012). To test whether this was the case for plants grown in our laboratory, we repeated the bacterial 16S rRNA gene profiling using universal primers PRK341F and PRK806R (**Supplementary Table S2**). Plants were grown under controlled conditions in Levington F2 compost to match the conditions in our previously published study (Worsley et al., 2020) and the other experiments reported here (for chemical analysis of the Levington F2 compost, see **Supplementary Table S3**). The number of OTUs detected in samples was greater in the bulk soil (an average of 3,258 ± 166.28 standard deviation across the three replicates) and rhizosphere (3,630.67 ± 1,413.12) compared to the endophytic compartment (1,144 ± 76.06). The endophytic compartment also had a reduced level of diversity, with the average Shannon diversity being 6.19 ± 0.18,

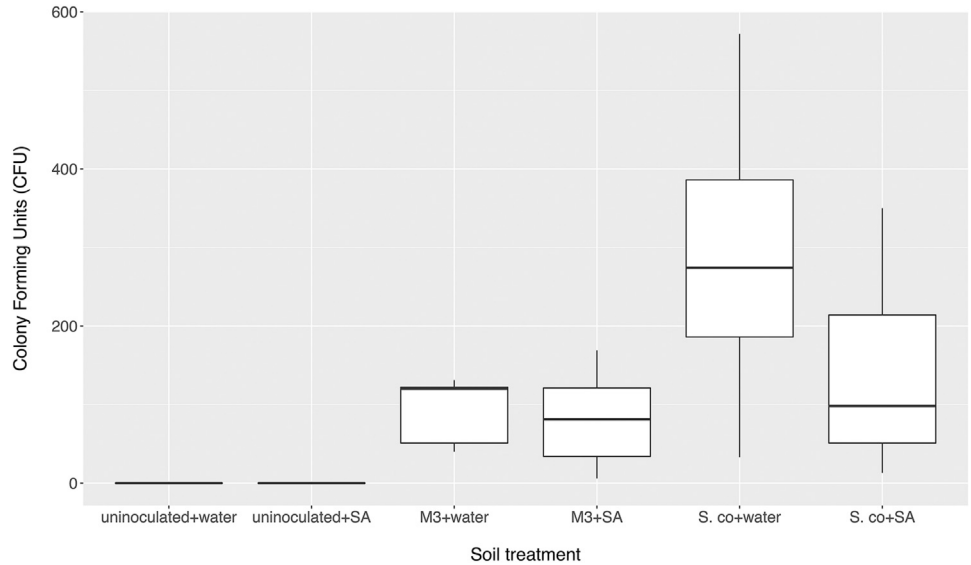

FIGURE 2 | Number of colony forming units (CFU) of inoculated *Streptomyces coelicolor* M145 or *Streptomyces* M3 strains returned from soil microcosms treated with either salicylic acid (SA) or distilled water after 10 days. Control microcosms weren't inoculated. *N* = 9 microcosms per treatment.

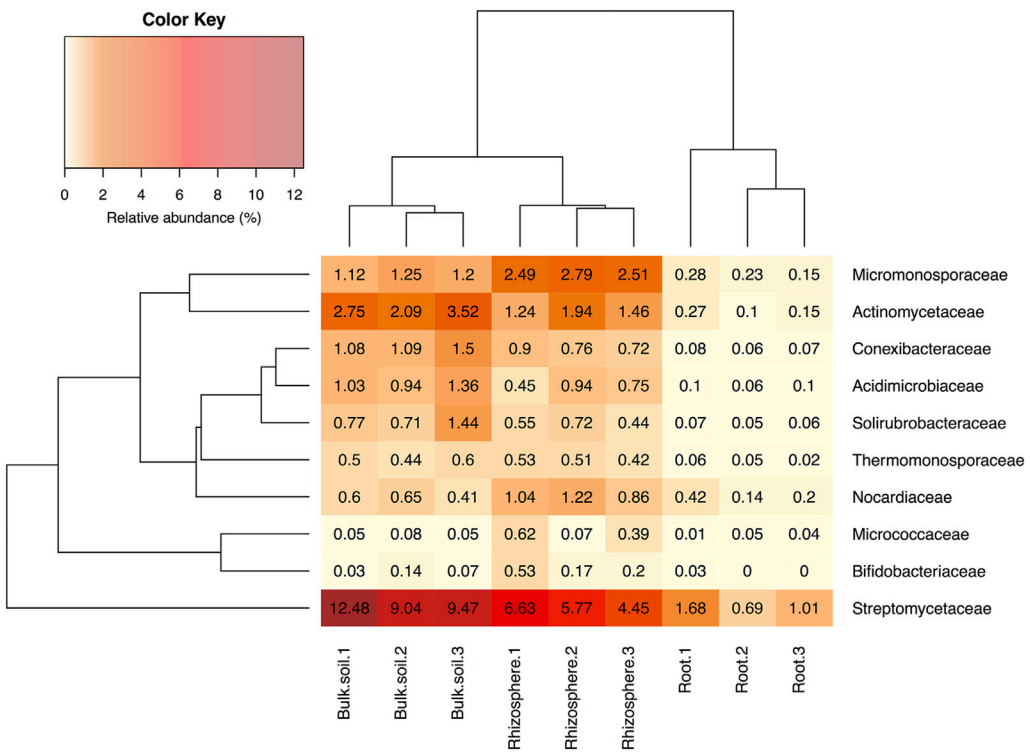

FIGURE 3 | The relative abundance of actinobacterial families in soil, rhizosphere and root compartments. *N* = 3 replicate plants in individual pots. Streptomycetaceae was the most abundant family of Actinobacteria in all three compartments. Clustering represents Bray Curtis dissimilarities.

6.18 ± 0.22 and 2.43 ± 0.17 in the bulk soil, rhizosphere and endosphere compartments, respectively. We found that, in agreement with published studies, *Streptomycetaceae* was the most abundant family of Actinobacteria in both the rhizosphere and root compartment of *A. thaliana* plants (**Figure 3**), making up 5.62% (±1.10% standard deviation) of the rhizosphere community

and 1.12% (±0.50%) of the endophytic community, respectively. Actinobacteria as a whole made up 15.15% (±1.47%) and 2.87% (±0.88%) of these compartments, respectively (**Figure 3** and **Supplementary Figure S4**). However, bacteria of the phylum Proteobacteria were found to dominate in all of the soil, rhizosphere and root compartments of the *A. thaliana* plants (**Supplementary Figure S4**) and were also significantly enriched in the endophytic compartment, relative to the surrounding soil and rhizosphere ($p < 0.05$ in Dunn's multiple comparison tests between root and rhizosphere and root and soil compartments) increasing to 91.22% (±0.74% standard deviation) of the root-associated community, compared to 38.85% (±9.80%) of the rhizosphere and 35.46% (±1.97%) of the soil community, respectively.

To test whether *Streptomyces* species metabolize *A. thaliana* root exudates we used $^{13}CO_2$ DNA-SIP (Radajewski et al., 2000; Dumont and Murrell, 2005; Haichar et al., 2016). Plants incubated with $^{13}CO_2$ fix the 'heavy' isotope of carbon which then becomes incorporated into carbon-based metabolites; many of these then leave the plant as root exudates. Bacteria feeding off labeled root exudates in the rhizosphere, or plant metabolites in the endosphere, will incorporate the ^{13}C into their DNA as they grow and divide. By separating ^{12}C- and ^{13}C-labelled DNA on a cesium chloride gradient and using universal primers (**Supplementary Table S2**) to amplify and sequence a variable region of the 16S rRNA gene from both heavy and light fractions, we can determine which bacteria are being fed by the plant in the rhizosphere and endosphere of *A. thaliana* (Neufeld et al., 2007; Haichar et al., 2016). *A. thaliana* plants were labeled by growing them ($n = 3$) in Levington F2 compost for 21 days. Plants were placed in sealed growth chambers in the presence of either $^{12}CO_2$ or $^{13}CO_2$ (**Supplementary Figure S5**). Unplanted pots were also incubated with $^{13}CO_2$ to account for autotrophic metabolism. Given the relatively short time frame of CO_2 exposure, ^{13}C was expected to be predominantly incorporated into plant metabolites rather than into plant cell wall material. Bacteria that incorporate ^{13}C into their DNA must therefore either be feeding on plant metabolites or fixing $^{13}CO_2$ autotrophically. After 21 days, total DNA was extracted from the rhizosphere, endosphere and unplanted soil compartments of the $^{12}CO_2$ or $^{13}CO_2$ incubated plants and heavy (^{13}C) and light (^{12}C) DNA were separated by density gradient ultracentrifugation. For the root samples, it was necessary to combine the three replicate samples into one sample for each of the CO_2 treatments before ultracentrifugation, due to low DNA yields. Heavy and light DNA fractions of each gradient were determined via qPCR and used for 16S rRNA gene amplicon sequencing (**Supplementary Figure S6**). An average (±standard deviation) of 2,109 ± 152.42, 5,083.33 ± 204.63, 2,252.67 ± 148.35, and 4,243 ± 605.49 OTUs were detected in the ^{12}C heavy, ^{12}C light, ^{13}C heavy and ^{13}C light fractions taken from rhizosphere samples, respectively. In the endophytic fraction samples, 999, 1,072, 1,000 and 2,570 OTUs were detected in the ^{12}C heavy, ^{12}C light, ^{13}C heavy and ^{13}C light fractions, respectively.

A principal coordinates analysis, using a Bray-Curtis dissimilarity matrix of the relative abundances of genera present in each of the different rhizosphere fractions suggested that certain genera had metabolized ^{13}C labeled host-derived resources, since ^{13}C and ^{12}C heavy fractions separated spatially on an ordination plot, indicating differences between the bacterial communities in these fractions (**Figure 4**). A permutational analysis of variance (PERMANOVA) analysis confirmed that fraction type had a significant effect on the community composition (permutations = 999, $R^2 = 0.80$, $p = <0.01$).

Several bacterial genera were found to be enriched in the heavy vs. the light fractions of the $^{13}CO_2$ unplanted control pots, suggesting that they may be capable of directly fixing CO_2 from the environment. This list included bacterial genera that are known autotrophs and, thus, these genera were not considered as utilizers of root exudates if they were enriched in the heavy fractions of rhizosphere or endosphere samples taken from ^{13}C planted pots (**Supplementary Table S4**). For example, several nitrifying bacteria were enriched in the heavy fractions of both the $^{13}CO_2$ treated plants and the unplanted controls; this included the genera *Nitrospira*, *Candidatus Nitrogena* and *Paracoccus*. A diverse range of CO_2 fixation strategies have been identified in nitrifying bacteria (Alfreider et al., 2018), and species in all of the aforementioned genera have been shown to use the Calvin-Benson-Bassham cycle to autotrophically fix carbon via the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO) (Bollmann et al., 2013; Kitzinger et al., 2018; Ye et al., 2020). Genes encoding the RubisCO enzyme have also been identified in several of the other genera that were enriched in the heavy fraction of control pots, including *Methylibium* and *Geobacter,* which have both been identified as key players in CO_2 fixation in rice paddies and agricultural soils (Wu et al., 2015; Xiao et al., 2021). The genus *Bacteroides* is also known to carry out direct carbon fixation during carbohydrate fermentation (Caldwell et al., 1969; Fischbach and Sonnenburg, 2011).

Disregarding possible autotrophs that were enriched in the heavy fractions of unplanted controls (**Supplementary Table S4**), a total of 28 genera showed an average of two-fold (or more) enrichment in the ^{13}C heavy (^{13}CH) fraction of rhizosphere samples, compared to both the $^{13}CO_2$ light fraction (^{13}CL) and $^{12}CO_2$ heavy fraction (^{12}CH), respectively, suggesting they had become labeled through the metabolism of root exudates (**Supplementary Table S4**; **Figure 5**). 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*) (**Supplementary Table S4**). The most enriched genus was *Pseudomonas*, which demonstrated a 64-fold enrichment in relative abundance between the ^{13}CH (8.63 ± 3.33% average relative abundance ± standard error) and ^{13}CL (0.13 ± 0.01%) fractions and a 23-fold enrichment between the ^{13}CH fraction compared to the ^{12}CH control (0.37 ± 0.06%) (**Figure 5**).

A total of 27 genera demonstrated more than a two-fold enrichment in the ^{13}CH fraction of the endophytic compartment vs. ^{13}CL and ^{12}CH fractions (**Figure 6**). The majority of these genera were also Proteobacteria (21 genera in total), with three genera belonging to the phylum Planctomycetes (*Blastopirellula, Pirellula* and *Gemmata*), one

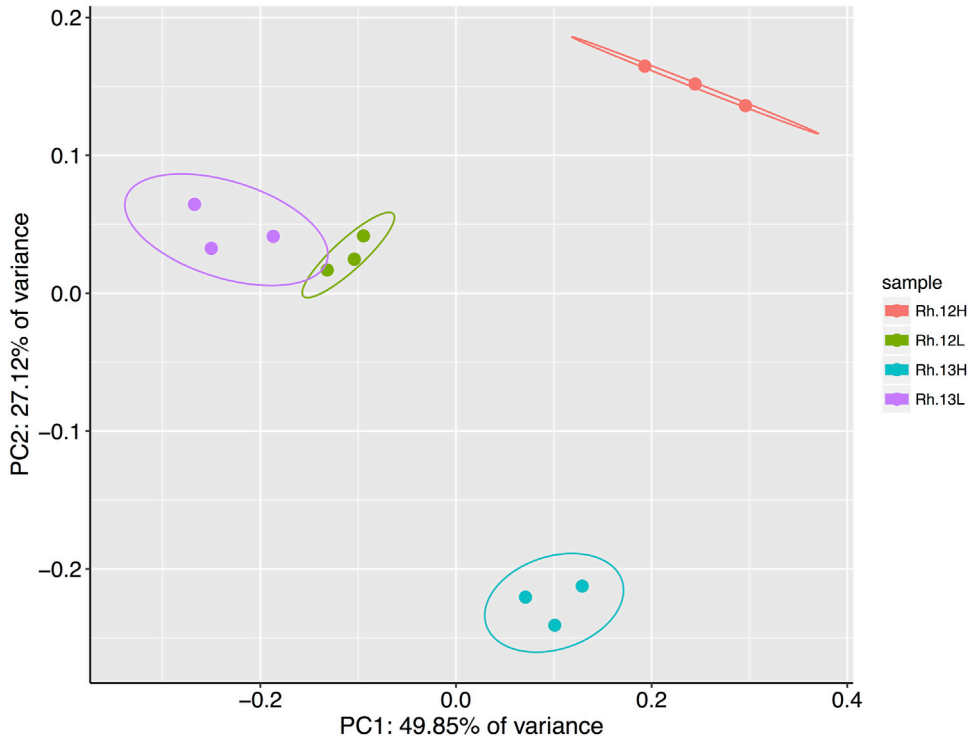

FIGURE 4 | Results of a principal coordinates analysis of Bray-Curtis dissimilarities between bacterial genera present in the heavy (H) and light (L) buoyant density fractions of rhizosphere samples. Samples were taken from plants incubated under either $^{12}CO_2$ (12L, 12H) or $^{13}CO_2$ (13L, 13H) treatments ($N = 3$ replicate plants under each treatment). Principle Component 1 and 2 explain 49.85 and 27.12% of the variance in Bray–Curtis dissimilarities, respectively, between fraction types. Ellipses represent 95% confidence intervals.

to the Firmicutes (*Clostridium*), one to the Chloroflexi (*Chloroflexus*), and one to the Actinobacteria (*Jatrophihabitans*) (**Supplementary Table S4**). 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%) (**Figure 6**). 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 (**Figure 6**). Comparing the two compartments, 15 out of the 28 genera were enriched by more than two-fold in the heavy fraction of the endosphere but not the rhizosphere samples, whereas 16 out of 29 genera were metabolizing exudates in the rhizosphere but not the endosphere. The remaining 13 genera were enriched in the heavy fractions of both the endophytic and rhizosphere compartments, suggesting that they are able to survive and make use of plant metabolites in both niches (**Supplementary Table S4**).

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 (**Figures 5** and **6** and **Supplementary Figure S7**; **Supplementary Table S4**). Taken together, these data suggest that, in compost, *Streptomyces* bacteria are outcompeted for root exudates by unicellular bacteria, particularly members of the phylum Proteobacteria. Proteobacteria were 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) (**Supplementary Figure S4**) and therefore may have the upper hand at the outset of competition (Scheuring and Yu, 2012). Accordingly, genera that were found to be metabolizing the greatest amount of exudates in the roots were also found to be enriched in the endophytic compartment compared to the surrounding soil (**Supplementary Figures S4 and S7**).

DISCUSSION

Although typically described as free-living soil bacteria, there is increasing evidence that *Streptomyces* species are effective at colonizing the rhizosphere and endosphere of plant roots where they can promote plant growth and provide protection against disease (Viaene et al., 2016; Rey and Dumas, 2017; Kim et al., 2019; Newitt et al., 2019). It has even been suggested that their diverse specialized metabolism and filamentous growth may

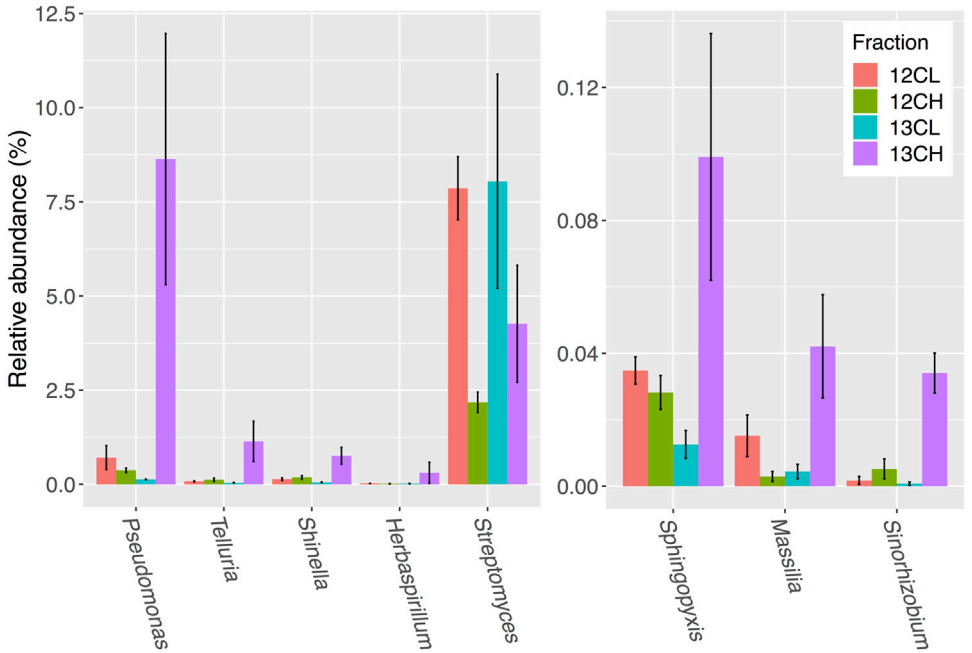

FIGURE 5 | Seven bacterial genera in the rhizosphere compartment of *A. thaliana* plants whose relative abundance demonstrated the greatest overall enrichment in the ^{13}CH fraction, relative to the ^{12}CH fraction, due to the metabolism of ^{13}C labeled root exudates. *Streptomyces* were not enriched but are included for comparison. 12CL/13CL and 12CH/13CH stand for buoyant density fractions representing the light (L) and heavy (H) fractions of DNA from $^{12}CO_2$ (12C) or $^{13}CO_2$ (13C) incubated plants, respectively. *N* = 3 replicate plants. Bars represent ± SE.

have evolved to give them a competitive advantage in this niche since it occurred ~50 million years after plants first colonized land (Chater, 2006; 2016). Plants certainly provide a good source of food in nutritionally poor soil environments, both through the release of root exudates but also by shedding more complex organic cell wall material as roots grow and senesce (Dennis et al., 2010). There is growing evidence that root exudates enable plants to attract bacteria and shape their microbiome (Sasse et al., 2018; Zhalnina et al., 2018; Huang et al., 2019). Consistent with this, the $^{13}CO_2$ DNA-SIP experiment identified several genera, particularly those in the phylum Proteobacteria, in both the rhizosphere and endosphere that became labeled with ^{13}C due to the metabolism of *A. thaliana* root exudates. Many of these genera have been shown to have a positive influence on plant fitness, suggesting that root exudates may be one mechanism by which beneficial bacteria can establish and be maintained within the plant root microbiome. For example, the genus *Pseudomonas* was highly enriched in the heavy fractions of the rhizosphere and endosphere samples of ^{13}C treated *A. thaliana* plants; this genus is well-known for producing many different bioactive and plant-growth-promoting molecules (Glick et al., 1997; Hernández-León et al., 2015; Mercado-Blanco et al., 2016; Raza et al., 2016) and certain species have been shown to promote root growth and induce systemic resistance to pathogenic infection in *A. thaliana* plants (Hase et al., 2003; Matilla et al., 2010; Zamioudis et al., 2013). Similarly, the genus *Massilia* colonizes the roots of many different plant species and has been shown to have plant growth promoting properties and antagonistic activity against phytopathogens *in vitro* (Ofek et al., 2012). The genus *Shinella* (a member of the Rhizobiaceae family) was also ^{13}C labeled in *A. thaliana* rhizosphere and endosphere samples. Members of this genus have previously been isolated from the root nodules of leguminous plants in which they are capable of fixing nitrogen and enhancing plant growth (An et al., 2006; Lin et al., 2008). Other genera in the Rhizobiaceae were also labeled in the $^{13}CO_2$ DNA-SIP experiment including *Ensifer*, *Sinorhizobium* and *Rhizobium*. Apart from inducing the formation of root nodules in leguminous plants, species in the genus *Rhizobium* have been shown to positively influence root development and overall plant biomass when interacting with *A. thaliana* plants (Zhao et al., 2017). Several of the non-proteobacterial genera that were identified as root exudate metabolisers in the rhizosphere and endosphere have been shown to be associated with plant species in other studies, although their role within the plant root microbiome is largely unknown. For example, species in the genus *Jatrophihabitans* (in the phylum Actinobacteria) are known to exist endophytically in several plant species and were labeled in the endosphere samples of ^{13}C treated *A. thaliana*-however, these species have not been characterized in relation to plant-growth-promoting traits (Madhaiyan et al., 2013; Gong et al., 2016).

Although we confirmed in this study that *Streptomyces* bacteria can survive using only *A. thaliana* root exudates *in vitro* (**Figure 4**), $^{13}CO_2$ DNA-SIP revealed that they were not ^{13}C-labelled in our experiments and were in fact likely outcompeted for plant metabolites in both the rhizosphere and endosphere compartments by the proteobacterial genera, that were abundant and labeled in both compartments (**Figure 5** and

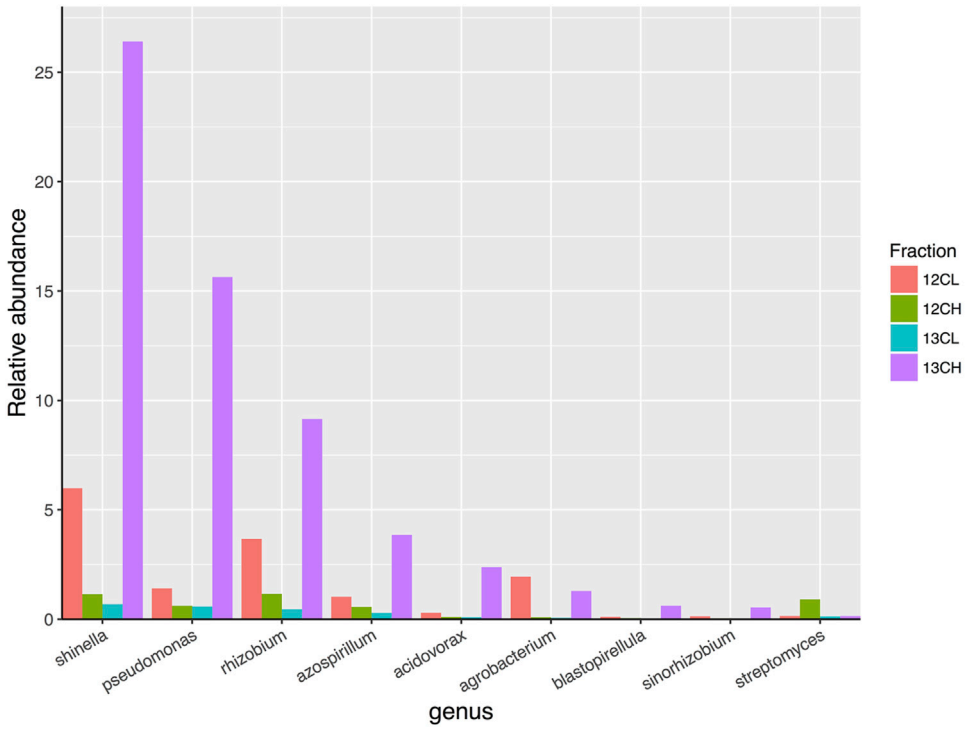

FIGURE 6 | The relative abundance of eight genera of bacteria that demonstrated the greatest overall enrichment between the ^{13}CH fraction and the ^{12}CH fraction due to the metabolism of ^{13}C labeled root exudates in the endophytic compartment of *Arabidopsis thaliana* plants. In comparison, the genus *Streptomyces* did not demonstrate enrichment between these fractions suggesting they did not metabolize ^{13}C-labelled root exudates. 12CL/13CL and 12CH/13CH are the buoyant density fractions representing the light/unlabelled (L) and heavy/labeled (H) fractions of DNA from $^{12}CO_2$ (12C) or $^{13}CO_2$ (13C) incubated plants, respectively.

Figure 6). Many proteobacteria are thought to be "r-strategists" that have the ability to thrive and rapidly proliferate in fluctuating environments where resources are highly abundant, such as in the rhizosphere and root-associated microbiome (Philippot et al., 2013). In contrast, species of *Streptomyces* typically exhibit slower growth and are competitive in environments where resource availability is limited, such as in the bulk soil (van der Meij et al., 2017). Thus, it is possible that proteobacteria were able to rapidly proliferate around and within the *A. thaliana* plant roots and outcompete the slower growing actinobacterial species, particularly those that were already present at lower abundance. Given their diverse metabolic capabilities, it is likely that streptomycetes are able to persist at low abundance by feeding on more complex organic polymers under conditions of high competition, including older, plant-originated organic material such as root cells and mucilage that were sloughed off before ^{13}C labeling commenced. *A. thaliana* also appears to exhibit a relatively small rhizosphere effect compared to other plant species (Schlaeppi et al., 2014; Bulgarelli et al., 2015), meaning that there is a weaker differentiation in terms of microenvironment and community composition between the rhizosphere and bulk soil, than there is between these two compartments and the endophytic niche, as observed in this study (**Supplementary Figure S4**). Thus, complex polymers may have been more readily available in the rhizosphere than exudates, particularly given the fact that the compost growth medium also contained a relatively high level of organic matter (91.08%, **Supplementary Table S3**).

We also tested whether *Streptomyces* strains *S. coelicolor* M145 and the *A. thaliana* endophyte *Streptomyces* M3 are specifically attracted to *A. thaliana* roots by a specific root exudate compound, salicylate, since a previous study suggested this molecule can modulate microbiome composition and specifically attract streptomycetes (Lebeis et al., 2015). However, we found no evidence to support this hypothesis. We found that *cpr5* mutant plants, which constitutively produce and accumulate salicylate, are colonized more readily by streptomycetes than wild-type plants, but we found no difference between the levels of *Streptomyces* root colonization in wild-type *A. thaliana* and salicylate-deficient *sid2-2* and *pad4* plants. Our data suggest that the *cpr5* effect is not directly due to salicylate, at least with the M145 and M3 strains we tested here. None of the eight *Streptomyces* strains we tested could use salicylate as a sole carbon source, and they did not respond chemotactically to salicylate or compete better in salicylate amended soil. We propose that the greater colonization efficiency observed in *A. thaliana cpr5* plants in this and a previous study (Lebeis et al., 2015) was not due to increased levels of salicylate, but more likely due to one or more of the pleiotropic effects of the *cpr5* mutation (Jing et al., 2007). The *cpr5* gene encodes a putative transmembrane protein (Kirik et al., 2001) that is part of a complex regulatory signaling network; its deletion results not only in the constitutive activation of SA biosynthesis genes, but

also stunted root and aerial growth, disrupted cellular organization, spontaneous cell death, early senescence in young plants, lesions on various tissue types, disrupted cell wall biogenesis, an increased production of ROS and high levels of oxidative stress (Bowling et al., 1994; Clarke et al., 2000; Kirik et al., 2001; Jing et al., 2007; Jing et al., 2008; Gao et al., 2011). Thus, it is possible that the altered cell wall composition and spontaneous cell death in *crp5* plants could have resulted in easier access to plant roots by streptomycetes, particularly as these bacteria are thought to enter roots in compromised areas, such as lesions and sites of wounding (Chater, 2006; Viaene et al., 2016). We also note that although that original study (Lebeis et al., 2015) reported that salicylate could be used as a sole C and N source by a *Streptomyces* endophyte strain, their Supplementary Material reported that sodium citrate was included in the growth medium used in these experiments and streptomycetes can grow using sodium citrate alone (**Supplementary Figure S1**).

In summary, we conclude that *Streptomyces* bacteria do not appear to be attracted to the rhizosphere by salicylic acid and did not feed on root exudates under the conditions presented in this study, because they were outcompeted by faster growing genera in the phylum Proteobacteria. Instead, it is likely that streptomycete bacteria were able to persist in the root microbiome by utilizing the organic matter present in the soil growth medium, in addition to components of the plant cells (such as cellulose) that are sloughed off during root growth. These ubiquitous soil bacteria are likely to be present in the soil as spores or mycelia when plant seeds germinate and root growth is initiated, enabling successful transmission between plant hosts. Although not tested in this study, it would be interesting to see whether *Streptomyces* spores germinate in the presence of plant root exudates. A greater understanding of the factors that attract beneficial bacteria to the plant root microbiome, and maintain them there, may have significant implications for agriculture. For example, it could inform efforts to develop and deliver effective plant growth-promoting agents and ensure they are competitive within the plant root niche. Indeed, studies have shown that organic matter amendments can improve the competitiveness and overall effectiveness of streptomycete biocontrol strains, presumably by providing them with an additional source of nutrients (Newitt et al., 2019). Clearly some *Streptomyces* strains can colonize the endosphere of plants and confer advantages to the plant host but it is not yet clear what advantage these bacteria gain from entering the plant roots, given that they must have to evade or suppress the plant immune system and are outcompeted for plant metabolites inside or outside the roots. Future work will address these questions and test the hypothesis that soil is a mechanism for vertical transmission of *Streptomyces* between generations of plants.

MATERIALS AND METHODS

Isolation and maintenance of *Streptomyces* strains. The *Streptomyces* strains L2, M2, M3, N1 and N2 (**Table 1**) were isolated from surface-washed *A. thaliana* roots in a previous study (Worsley et al., 2020). Genome sequences were also generated for each of these isolates, as well as for three strains of *Streptomyces lydicus*, one of which was isolated from the commercial biocontrol product Actinovate, while the remaining two (ATCC25470 and ATCC31975) were obtained from the American Type Culture Collection (**Table 1**). In the present study, *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) (**Supplementary Table S5**). Strains were spore stocked as described previously (Kieser et al., 2000).

Generating eGFP-Labelled *Streptomyces* Strains

Plasmid pIJ8660 (**Supplementary Table S1**) containing an optimized eGFP gene and an aac apramycin resistance marker (Sun et al., 1999) was altered using molecular cloning of the constitutive *ermE** promotor to drive eGFP expression. Primers ermEKpnFwd and ermENdeRev (**Supplementary Table S1**) were used to amplify *ermEp** from the plasmid pIJ10257 (**Supplementary Table S1**) (Hong et al., 2005). The PCR product was subsequently purified and then digested with the restriction enzymes KpnI and NdeI, before being ligated into the same sites in pIJ8660. The resulting plasmid, called pIJ8660/*ermEp** (**Supplementary Table S1**), was conjugated into the *Streptomyces* strains M3 and *S. coelicolor* M145 (**Table 1**) as described previously (Kieser et al., 2000). Exconjugants were selected and maintained on SFM agar plates containing 50 mg mL^{-1} apramycin.

Plant root colonization assays in soil. Wild-type *A. thaliana* Col-0 as well as the mutant plant lines *cpr5*, *pad4*, and *sid2-2* were obtained from the Nottingham *Arabidopsis* Stock Centre (**Table 1**). Seeds were sterilized by placing them in 70% (v/v) ethanol for 2 min, followed by 20% NaOCl for 2 min and then washing them five times in sterile dH_2O. Spores of either *S. coelicolor* M145-eGFP or *Streptomyces* M3-eGFP (**Table 1**) were used as inoculants and were pre-germinated in 2×YT (**Supplementary Table S5**) at 50°C for 10 min (Kieser et al., 2000). Uninoculated 2×YT was used as a control. Surface sterilized seeds were placed into 500 µl 2×YT containing 10^6 spores mL^{-1} of pre-germinated *Streptomyces* spores; these were mixed for 90 min on a rotating shaker before being transferred to pots of sieved Levington F2 seed and modular compost, soaked with distilled H_2O. Another 1 mL of pre-germinated spores (or 2×YT for control pots) was pipetted into the soil to a depth of approximately 2 cm below the seed. Pots were incubated at 4°C for 24 h, then grown for 4 weeks under a 12-h photoperiod. Six replicate plants were grown for each plant genotype and each *Streptomyces* strain.

Re-isolation of *Streptomyces* bacteria from roots

In order to re-isolate tagged strains from the roots of the different *A. thaliana* genotypes we used a previously established method (Lebeis et al., 2015). Briefly, plants were taken aseptically from pots and their roots were tapped firmly to remove as much adhering soil as possible. Root material was washed twice in sterile PBS-S buffer (**Supplementary Table S5**) for 30 min on a shaking platform and then any remaining soil particles were removed with sterile tweezers. Cleaned roots were then transferred to 25 mL of fresh PBS-S and sonicated for 20 min to remove any residual material still attached to

the root surface; this ensured that any remaining bacteria were either present in the endophytic compartment or were very firmly attached to the root surface ("the rhizoplane"). Cleaned roots were weighed, then crushed in 1 mL of sterile 10% (v/v) glycerol. 100 µl of the homogenate was plated onto three replicate soya flour plus mannitol agar plates containing 50 µg mL^{-1} apramycin to select for inoculated streptomycetes (**Supplementary Table S5**). Plates also contained 5 µg mL^{-1} nystatin and 100 µg mL^{-1} cycloheximide to inhibit fungal growth. Agar plates were incubated at 30°C for 5 days, before the number of apramycin-resistant *Streptomyces* colony forming units (cfu) were counted. This method has been used previously in other studies (Bonaldi et al., 2015; Chen et al., 2016) to estimate the root colonization dynamics of streptomycetes. Counts were converted to cfu per gram of root tissue and log-transformed to normalize residuals. Data were analyzed via ANOVA and post-hoc Tukey's Honest Significant Difference (HSD) tests.

Testing for sole use of carbon and nitrogen sources

The *A. thaliana* endophyte *Streptomyces* strains L2, M2, M3, N1 and N2, as well as the *S. lydicus* strains ATCC25470, ATCC31975 and Actinovate (**Table 1**), were streaked onto minimal agarose medium plates supplemented with either their preferred carbon source as a positive control, 3.875 mM sodium citrate, or 0.5 mM salicylic acid (SA) as a carbon source (**Supplementary Table S5**). Preferred carbon sources were 5 g L^{-1} of mannitol for strains N2 and M2; 5 g L^{-1} maltose for strains M3, N1, *S. lydicus* 25470 and *S. lydicus* Actinovate; 5 g L^{-1} sucrose for L2; 10 g L^{-1} glucose for *S. lydicus* 31975. Plates with no carbon source were used as a negative control. All strains were plated in triplicate onto each type of media and incubated for seven days at 30°C before imaging. *S. coelicolor* M145 was not tested as it can use agarose as a sole carbon source (Stanier, 1942; Temuujin et al., 2012). Agarose was used as a gelling agent as streptomycetes can grow on impurities that are present in agar.

Screening for salicylate metabolism genes in the genomes of *Streptomyces* isolates

To identify whether the nine different streptomycetes carried homologues to known SA degradation genes, all experimentally verified pathways involving SA (or salicylate) degradation were identified using the MetaCyc database (http://www.metacyc.org/) and amino acid sequences of characterized genes involved in each of the five pathways were then retrieved from UniProt (**Supplementary Table S1**). Protein sequences were used to perform BLASTP searches against predicted Open Reading Frames (ORFs) for each genome-sequenced streptomycete. Previously generated sequences for N1, N2, M2, M3, L2, *S. lydicus* 25470, *S. lydicus* 31975 and *S. lydicus* Actinovate were used (Worsley et al., 2020) as well as the published genome sequence of *S. coelicolor* (Bentley et al., 2002). The results of the best hit (% identity and % query coverage) are reported.

SA as a chemoattractant

To test whether streptomycetes grew toward SA, 4 µl of spores (10^6 spores mL^{-1}) of each streptomycete (**Table 1**) were pipetted onto the centre of SFM agar plates. 40 µl of either 1 mM or 0.5 mM filter-sterilized SA was inoculated onto 6 mm 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 and 0.5 mM solutions had a final concentration of 0.2% (v/v) and 0.1% (v/v) 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. All strains and disc type pairings were plated in triplicate and were incubated for seven days at 30°C before imaging.

Enumeration of Bacteria from Soil Microcosms Following Exogenous Application of SA

Levington F2 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 a spore solution (10^7 spores mL^{-1}) of either *S. coelicolor* M145-eGFP or *Streptomyces* M3-eGFP (**Table 1**), suspended in dH_2O or 0.5 mM SA. Spores of eGFP-tagged M3 or M145 were used to align with *in vivo* plant colonization experiments (outlined above). There were nine replicate wells for each treatment. Well-plates were placed under a 12-h photoperiod 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) in addition to 10 µg mL^{-1} nystatin and 100 µg mL^{-1} cyclohexamide (to repress fungal growth). CFU of the *Streptomyces* inoculum were then enumerated on 10^{-2} dilution plates after 4 days to assess whether SA affected the competitiveness of strains in soil. A generalized linear model (GLM) with a negative binomial distribution was generated, using the package MASS in R 3.2.3 (R Core Team, 2017), to model the effect of strain (*S. coelicolor* M145-eGFP or *Streptomyces* M3-eGFP), soil treatment (wetting with SA or dH2O), and their interaction term (soil treatment) on the number of bacterial cfu returned from soil wells. Likelihood ratio tests were used to establish the significance of terms in the model.

Root Exudate Collection and Growth of Streptomycetes

To test whether streptomycetes were able to utilize other compounds in root exudates more generally, root exudates were isolated from *A. thaliana* and used as a growth medium for the *Streptomyces* strains (**Table 1**) that we previously isolated from *A. thaliana* plants growing in a compost system (Worsley et al., 2020). Seeds of *A. thaliana* Col-0 were sterilized (as described above) and germinated on MSk agar (**Supplementary Table S5**). After 7 days of growth at 22°C under a photoperiod of 12 h light/12 h dark, seedlings were

transferred to 12 well plates, containing 3 mL of liquid MSk (**Supplementary Table S5**, 0% w/v sucrose) in each well. Seedlings were then grown for a further 10 days before being washed and transferred to new wells containing 3 mL of sterile water. After 5 days plant material was removed and the liquid from each well was filter-sterilized and added to sterile agarose (0.8% w/v) to make solid growth medium plates. Spores of previously isolated *Streptomyces* isolates (**Table 1**) 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.

$^{13}CO_2$ Stable Isotope Probing

Seeds of *A. thaliana* Col-0 (**Table 1**) were sterilized, as described above, before being sown singly into pots containing 100 mL sieved Levington F2 compost, soaked with dH_2O. These were placed in the dark at 4°C for 48 h, after which they were transferred to short-day growth conditions (8 h light/16 h dark) at 22°C for 32 days before exposure to CO_2 treatments. Each plant was placed into an air-tight, transparent 1.9 L cylindrical tube. Three plants were exposed to 1,000 ppmv of $^{12}CO_2$ and three plants were exposed to 1,000 ppmv of $^{13}CO_2$ (99%, Cambridge isotopes, Massachusetts, United States). Three unplanted controls containing only Levington F2 compost were additionally exposed to 1,000 ppmv of ^{13}C labeled CO_2 to control for autotrophic consumption of CO_2 by soil microbes. CO_2 treatments took place over a period of 21 days. CO_2 was manually injected into tubes every 20 min over the course of the 8 h light period, to maintain the CO_2 concentration at ~1,000 ppmv; CO_2 concentration was measured using gas chromatography (see below). At the end of the light period each day, tube lids were removed to prevent the build-up of respiratory CO_2. Just before the next light period, tubes were flushed with an 80%/20% (v/v) nitrogen-oxygen mix to remove any residual CO_2 before replacing the lids and beginning the first injection of CO_2.

Gas Chromatography

The volume of CO_2 to be added at each injection in the SIP experiment was determined by measuring the rate of uptake of CO_2 over 20 min every 4 days. This was done using an Agilent 7890A gas chromatography instrument with a flame ionization detector and a Porapak Q (6 ft × 1/8″) HP plot/Q (30 m × 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 min, with CO_2 having a retention time of 3.4 min. 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).

Sampling and DNA Extraction from 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. Roots were then processed to collect the rhizosphere soil and endophytic samples according to a published protocol (Bulgarelli et al., 2012). For the planted pots, roots were tapped until only the 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 (**Supplementary Table S5**) and washed on a shaking platform at top speed for 30 min before being transferred to fresh PBS-S. Used PBS-S from the first washing stage was centrifuged at 1,500 x g for 15 min, 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 min before removing any remaining soil particles with sterile tweezers. Finally, the cleaned roots were transferred to fresh PBS-S and sonicated for 20 min in a sonicating water bath (Bulgarelli et al., 2012). Root samples for each plant were then snap frozen and stored at −80°C. Each root 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-homogenization of the root material by grinding in liquid nitrogen before adding lysis buffer, an extended incubation time (10 min) 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 root samples it was necessary to combine the three replicate samples for each CO_2 treatment due to low DNA yields. 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 (Reichert Analytical Instruments, NY, United States) in order to check for successful gradient formation. DNA was precipitated from fractions (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 2x Sybr Green Jumpstart Taq Ready-mix (Sigma Aldrich), 0.125 µl of each of the primers PRK341F and MPRK806R (**Supplementary Table S2**), 4 µl of 25 mM MgCl2, 0.25 µl of 20 µg μl^{-1} Bovine Serum Albumin (BSA, Roche), and 7 µl dH2O. Sample DNA, standards (a dilution series of the target 16S rRNA gene at known molecular quantities), and negative controls were quantified in duplicate. Reactions were run under the following conditions: 96°C for 10 min; 40 cycles of 96°C for 30 s, 52°C for 30 s, and 72°C for 1 min; 96°C for 15 s; 100 cycles at 75–95°C for 10 s, ramping 0.2°C per cycle. Reactions were performed in 96-well plates (Bio-Rad). The threshold cycle (CT) for each sample was then converted to target molecule number by comparing to CT values of a dilution series of target DNA standards. Fractions spanning the peaks in 16S rRNA gene copy number were identified and equal quantities of these were combined to create a "heavy" buoyant density (labeled) 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 (**Supplementary Table S2**), 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, United States. This generated a total of 3′699′266 reads, with an average of 127,560.90 ± 85,376.35 (standard deviation) reads per sample. Sequence data were then processed at MR DNA using their custom pipeline (Dowd et al., 2008a; Dowd et al., 2008c). 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 denoized, 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). A post processing algorithm generated best-hit files with *E*-values < 10e−114 and bit scores > 400. The identities of all hits were >98%. These parameters have been previously evaluated to enable reliable identification at the genus level (Dowd et al., 2008b). Plastid-like sequences and singletons were removed from downstream analysis. A total of 1′938′580 reads remained after processing, with an average of 66,847.59 ± 40,423 (standard deviation) reads per sample. OTU abundances were converted to relative abundances in all downstream analyses to control for the variation in library sizes across samples. All data received from MR DNA were then further processed and statistically analyzed using R 3.2.3 (R Core Team, 2017), using the packages tidyr and reshape (for manipulating data-frames), ggplot2 and gplots (for plotting graphs and heatmaps, respectively), vegan (for calculating Bray–Curtis dissimilarities, conducting principle coordinate and PERMANOVA analyses, calculating Shannon diversity, and for generating heatmaps) and ellipse (for plotting principal coordinate analysis). All the 16S rRNA gene amplicon sequences have been submitted to the European Nucleotide Archive (ENA) database under the study accession number PRJEB30923.

Chemical Analysis of Levington Compost

Soil pH, organic matter content (%) as well as the levels of phosphorus, potassium and magnesium (all in mg/kg), was measured by the James Hutton Institute Soil Analysis Service (Aberdeen, United Kingdom). To quantify inorganic nitrate and ammonium concentrations a KCl extraction was performed in triplicate, whereby 3 g of soil was suspended in 24 mL of 1 M KCl and incubated for 30 min, with shaking, at 250 rpm. To quantify ammonium concentration (g/kg) an indophenol blue method was used (as described in Verdouw et al., 1978). For nitrate concentration (in g/kg) vanadium (III) chloride reduction followed by chemiluminescence was used (as described in Braman and Hendrix, 1989).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB30923.

AUTHOR CONTRIBUTIONS

SW, MM, SP, BW, JM, and MH designed the research. SW, JM, and MH wrote the manuscript with comments from all other authors. SW performed the strain isolation and plant colonization experiments. SW performed the DNA stable isotope probing experiments with help from MM. SP carried out the chemical analysis of compost. SW performed all statistical analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.686110/full#supplementary-material

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