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Root-Associated *Streptomyces* Isolates Harboring *melC* Genes Demonstrate Enhanced Plant Colonization

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ABSTRACT

Streptomyces assemble into the internal, root endophytic compartment of a wide variety of plants grown in soils worldwide, suggesting their ability to survive during root microbiome assembly. A previous study found that among four nonpathogenic, root-isolated *Streptomyces* strains (303, 299, CL18, and 136), only 303 and 299 colonized endophytic root tissue of the majority of *Arabidopsis thaliana* roots when inoculated with 34 other bacterial isolates. Here we demonstrate that 303 and 299 also colonize significantly more in singly inoculated *A. thaliana* seedlings. The genomes of melanin-producing 303 and 299 each contain two copies of the gene encoding tyrosinase (*melC2* and *melD2*), an enzyme necessary for melanin biosynthesis in *Streptomyces*. These genes were not found in the genomes of 136 or CL18. Tyrosinase activity was detected in 303 and 299 whole cell and supernatant protein extracts, suggesting functional intracellular and extracellular enzymes. Because tyrosinase oxidizes phenolic compounds and *Streptomyces* colonization of *A. thaliana* appears to be influenced by the phenolic compound salicylic acid (SA), we

measured direct sensitivity of *Streptomyces* isolates to the phenolic compounds catechol, ferulic acid (FA), and SA in vitro. While both 303 and 299 showed higher numbers of surviving colonies than CL18 and 136 in the presence of catechol, only 303 demonstrated a higher number of surviving colonies when isolates were challenged with FA and SA. Finally, when seedlings were singly inoculated with a collection of related plant-associated *Streptomyces* isolates, colonization was significantly higher in isolates possessing two tyrosinase gene copies than isolates with zero or one gene copy. Overall, we describe a connection between microbial tyrosinase activity and increased seedling colonization of nonpathogenic *Streptomyces* isolates in *A. thaliana*. We propose tyrosinase activity in *Streptomyces* partially protects against harmful plant-produced phenolic compounds as they transition into an endophytic lifestyle.

Keywords: bacteriology, endophytes, genomics, microbiome, molecular biology

During plant microbiome assembly, both hosts and microbes drive the complicated and interwoven mechanisms that result in

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community composition (Coyte et al. 2015). The microbial members of these communities likely scavenge resources and nutrients from their surroundings (Hibbing et al. 2010; Wei et al. 2015), withstand abiotic and biotic assault (Jousset et al. 2011; Lareen et al. 2016), and/or act aggressively toward competing microbes (Fiegna and Velicer 2005). Microbiome assembly is likely influenced by cooperation and competition between microbes vying for niches within the root. Additionally, negotiation of the plant immune system requires specific microbial abilities and potentially host accommodation. Rhizosphere and internal, root endophytic compartment (EC) microbiomes are assembled through plant-specific recruitment of subsets of the soil microbial reservoir, resulting in host-specific microbiome assemblages that are often rich in certain taxa and deplete in others (Sasse and Northern 2018; Zhelnina et al. 2018). While shifts in community composition may result in a net benefit or cost to the host and other microbes (Lakshmanan et al. 2014), it is still critical to define the finer scale

influence of individual microbiome members on host health (Coyte et al. 2015; van der Heijden and Hartmann 2016). Understanding incentives for specific host-microbiome establishment will facilitate beneficial community manipulation to improve plant health and growth through defined agricultural practices (Lareen et al. 2016; Sheth et al. 2016).

Deciphering the mechanisms that promote successful colonization of individual microbial strains within a complex microbial inoculum is essential for the development of successful biological products to improve plant health. Although *Streptomyces* are not a dominant root-associated family, they are consistently enriched within the roots of various plant species compared with surrounding soil inoculum and are regarded for their metabolic potential (Bonaldi et al. 2015; Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012; Peiffer et al. 2013; Seipke et al. 2012; Viaene et al. 2016; Yeoh et al. 2016), which provides us with the opportunity to identify novel molecular mechanisms that contribute to their colonization success. While strains of *Streptomyces scabiei* and *S. ipomoeae* are plant pathogens (Bignell et al. 2014), most other strains are nonpathogenic and consistent root microbiome members. Many *Streptomyces* contribute to agriculturally important traits, such as drought resistance, improved plant growth promotion, and disease resistance through biocontrol capabilities (Fitzpatrick et al. 2018; Viaene et al. 2016; Xu et al. 2018). However, it is still necessary to tease apart unique mechanisms that facilitate *Streptomyces* root colonization. Here we define *Streptomyces* strain-specific mechanisms that might potentiate survival advantages during root microbiome assembly.

Previous experiments inoculated axenic *Arabidopsis thaliana* with a synthetic community of 38 taxonomically diverse bacterial isolates, which included four *Streptomyces* isolates, and found that their colonization of 6- to 8-week-old *A. thaliana* roots differed (Lebeis et al. 2015). Specifically, two *Streptomyces* isolates, 303 and 299, were found with significantly greater abundance in the root EC than in inoculum and were thus indicated as “EC-enriched,” while the other two, CL18 and 136 were not (Lebeis et al. 2015). Root EC colonization by these strains was influenced by salicylic acid (SA), a phenolic compound, which inhibits microbial growth (Bosund et al. 1960; Yang et al. 2015), acts as a plant defense phytohormone (Rivas-San Vicente and Plasencia 2011; Studham and MacIntosh 2012), and is measurable in root tissue, seedlings, and exudates (Chaparro et al. 2013; Lebeis et al. 2015; Strehmel et al. 2014; Zhalnina et al. 2018). Isolate 136 colonized significantly better in *pad4* plants, which are unable to trigger SA accumulation, while 303 displayed significantly increased levels of root colonization in plants sprayed with exogenous SA (Lebeis et al. 2015). Thus, *Streptomyces* colonization appears to be influenced by at least one phenolic compound present in the root–soil interface. However, it was unclear if SA influence was direct or indirect, acting through the plant immune system. Here we explore distinct phenotypic and genomic characteristics of these four nonpathogenic, *A. thaliana* root-associated *Streptomyces* strains. While recent studies established that plant-associated *Streptomyces* do not use SA as a carbon source during colonization (Worsley et al. 2019), here we test the hypothesis that SA and other phenolic compounds in the root/soil interface provide selective pressure during root microbiome community assembly. We investigate the strain-specific kinetics and products of tyrosinase an enzyme present in the two root EC-enriched strains, 303 and 299, but not CL18 or 136. Further, we suggest that inhibiting this enzyme negatively influences protection against phenolic compounds during seedling colonization. These studies present a unique opportunity to elucidate microbial determinants of seedling colonization for root-associated strains within a single genus.

Streptomyces fall within the phylum *Actinobacteria*, a taxonomic group with vast and varied metabolic potential (Barka et al. 2016). We propose that one beneficial product for plant-associated *Streptomyces* strains during colonization is melanin, a pigment ranging in color from tan to black, which is also produced by other bacterial taxa, fungi, plants, insects, and mammals (Eisenman and Casadevall 2012; Emami et al. 2017; Manivasagan et al. 2013; Valverde et al. 1995; Zhang et al. 2017). In bacteria, the polymer is produced in various forms including eumelanin, which is produced during tyrosinase oxidation and is black-brown, pheomelanin, which is yellow-red and produced via cysteinylolation of dihydroxyphenylalanine (DOPA), and the less studied allomelanin (Plonka and Grabacka 2006). These melanins can shuttle electrons or act as final electron acceptors (Plonka and Grabacka 2006). While function and synthesis vary by organism, melanins are generally hypothesized to provide protection from stresses such as reactive oxygen species, antibiotics, and antimicrobial peptides (Brenner and Hearing 2008; Manivasagan et al. 2013; Yang and Chen 2009), thereby providing a potential survival advantage (Nosanchuk and Casadevall 2003). In the root pathogen *S. scabiei*, mutants that lose melanin production exhibit decreased virulence, although this phenotype often co-occurred with decreased production of the virulence factor thaxtomin A (Beausejour and Beaulieu 2004). Therefore, it remains unclear what role melanin might play during root colonization with nonpathogenic *Streptomyces*.

The extracellular production of melanin in *Streptomyces* is frequently observed and conferred by the *melC* operon, which contains two genes, *melC1* and *melC2*. *melC2* encodes a tyrosinase enzyme critical for oxidizing compounds at multiple steps during melanin production from tyrosine (Bernan et al. 1985; Katz et al. 1983; Lee et al. 1988). *melC1* encodes a helper protein that adds the required copper ions for tyrosinase function, and contains a secretion signal that facilitates the exporting of both MelC1 and MelC2 proteins into the extracellular space, where the MelC2 enzyme participates in melanin production (Chen et al. 1992; Leu et al. 1992). A previous study discovered that a collection of melanin producing *Streptomyces* isolates contained two predicted copies of the *melC* operon, each containing the two required genes (Yang and Chen 2009). The second copy of the operon (termed *melD*) resulted in an intracellular enzyme and phenolic compounds oxidation within the cell (Yang and Chen 2009). While *melD* was observed in strains without *melC*, the opposite was not true (Yang and Chen 2009). Yang and Chen observed that strains with only *melD* lacked melanin production, confirming that *melC* confers extracellular melanin production (Yang and Chen 2009). Here we describe that *Streptomyces* isolates 303 and 299, which are both capable of producing melanin also possess a predicted *melC* and a predicted *melD* operon and exhibit increased resistance to phenolic compounds. The experimental system we use allows us to determine the potential survival advantage of *mel*-harboring isolates via hypothesized protective tyrosinase or melanin activities to enhance the opportunity to colonize plants, which we test on four *Streptomyces* strains isolated from a different plant host species. Advancing our understanding of how *Streptomyces* spp. colonize *A. thaliana* will provide future opportunities to understand their activities in root microbiomes and determine if they manipulate microbiome composition to improve plant health, growth, and ultimately yield.

MATERIALS AND METHODS

***Streptomyces* culture preparation.** *Streptomyces* isolates were grown in lysogeny broth (LB) (Bertani 1951) at 30°C with shaking at 150 rpm for 4 to 7 days. Cultures were vortexed vigorously for 5 s

and beaten with 3 mm glass beads for 2.5 min to disrupt bacterial aggregates. A spectrophotometer measured the optical density at 600 nm (OD_{600}) and cultures were normalized to an OD_{600} of 0.01. One hundred microliters of all normalized isolate resuspensions were plated on LB solid medium, incubated at 28°C for 4 to 7 days, and colony forming units (CFUs) were counted. Inoculum ranged from 1×10^2 to 3×10^4 CFU/ml. LB medium was used for initial inoculation of many of our assays due to the consistent size of flocculants and pigment production that two of our *Streptomyces* strains made on solid and in liquid media that made it possible to differentiate the isolates from one another (Fig. 1A). Additionally, because it is a rich medium, we could easily detect contamination with other faster growing bacteria.

Pigment extraction. Pigment extraction from bacterial strains was adapted from Drewnowska et al. (2015). Specifically, 200 ml of cultures of 303, 299, CL18, and 136 was split between four 50-ml sterile conical tubes each and centrifuged for 15 min at $3,200 \times g$. Supernatants of each isolate were transferred to two 100-ml glass bottles. The pH of the supernatants was adjusted to 2.0 via addition of 1 M HCl. Samples were incubated at room temperature for 1 week in the dark. Following incubation, the acidified supernatants were boiled in the glass bottles for 1 h. Cooled supernatants from each isolate were transferred to four 50-ml conical tubes and

centrifuged for 15 min at $3,200 \times g$. After supernatants were removed, 303, 299, and CL18 had approximately a 1-ml pellet remaining in each tube while 136 had no evident pellet. Pellets were resuspended and combined into a single 15-ml tube per isolate. The two tubes were then centrifuged again for 7 min at $3,200 \times g$ and supernatants were discarded. Pellets were washed and centrifuged for 7 min at $3,200 \times g$ three times in 15 ml of 0.1 M HCl and a final time in 15 ml of water. After each wash, supernatants were discarded. After washing, 10 ml of absolute ethanol was added to each of the 15-ml tubes containing the pellets and resuspended. The tubes were placed in a boiling water bath for 10 min and then incubated at room temperature for 1 day. Following incubation, the suspensions were centrifuged for 7 min at $3,200 \times g$ and the supernatant was discarded. The pellets were washed twice with absolute ethanol and centrifuged for 7 min at $3,200 \times g$ between washings. After the second wash, the pellets were allowed to air dry. The pellets for 303 and 299 cultures were brown while the pellet from CL18 culture was orange.

Liquid-chromatography mass spectrometry (LC-MS). Pigment pellets were digested following the protocol established by Ito and Wakamatsu (1998). Briefly, approximately 1-mg portions of the dried pigments were weighed and transferred to glass vials (4 dr) for digestion. The solid samples were suspended in 100 μ l of

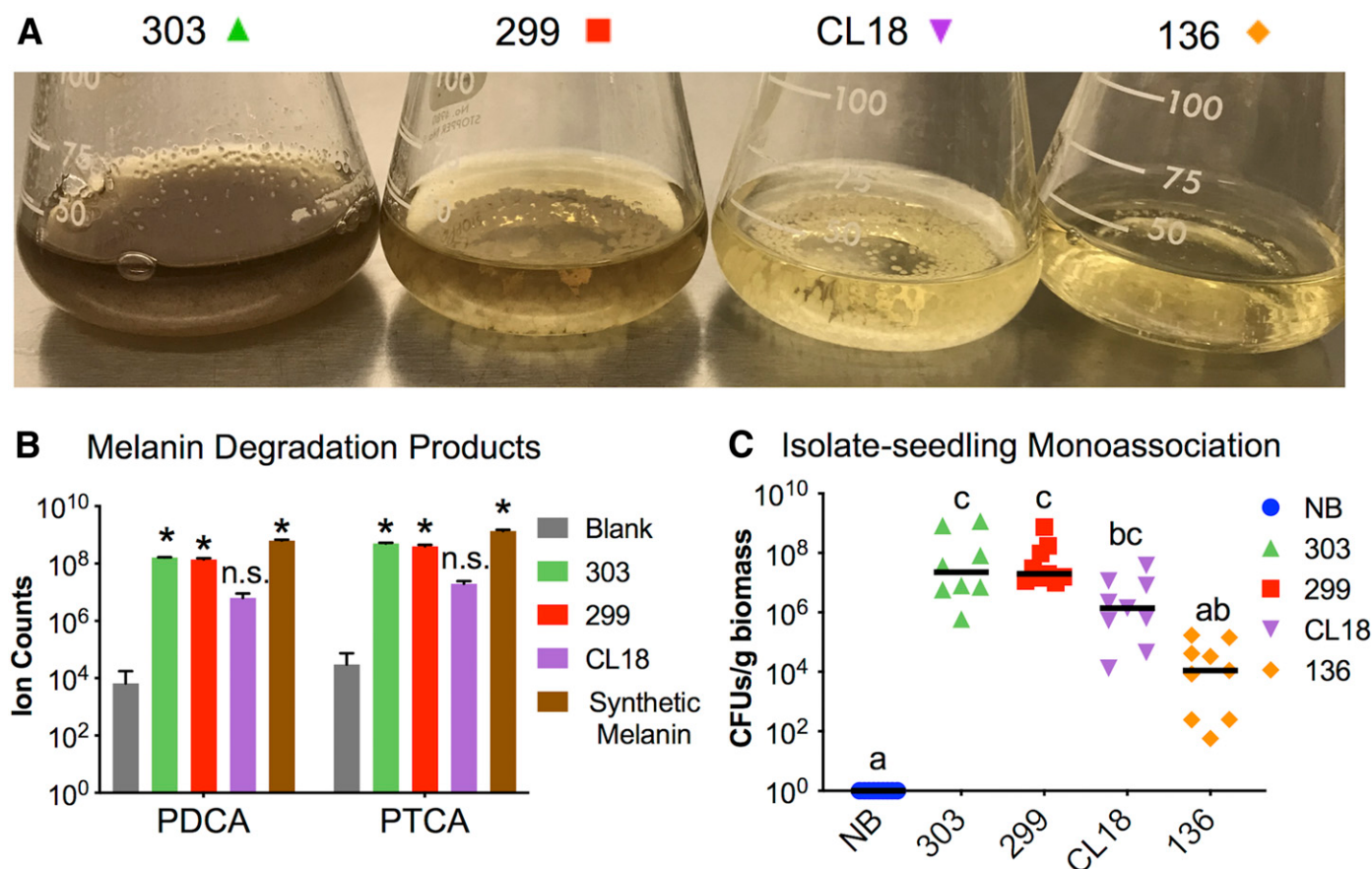


Fig. 1. *Streptomyces* strains 303 and 299 produce a pigment consistent with synthetic melanin. **A**, Strains 303 (far left) and 299 (middle left) liquid cultures produce an extracellular pigment in lysogeny broth medium. **B**, Liquid chromatography-mass spectrometry of pigmented bacterial extracts indicate degradation components consistent with melanin (gray bars, $n = 13$), 303 extracts (green bars, $n = 3$), 299 extracts (red bars, $n = 3$), CL18 extracts (purple bars, $n = 3$), and synthetic melanin control (brown bars, $n = 3$). Ion counts normalized to mass digested means are displayed with standard deviation indicated by error bars. * Indicates significantly different from the blank controls ($P < 0.0001$), two-way analysis of variance with Dunnett's multiple comparison test. **C**, Axenic 7 day-old Col-0, wild-type seedlings were singly inoculated with each *Streptomyces* isolate (no bacteria control [NB, blue circles], 303 [green triangles], 299 [red squares], CL18 [purple triangles], or 136 [orange diamonds], $n = 6$ to 9) and grown for 14 days when seedling colonization level was determined. Different letters indicate statistical differences using a Kruskal-Wallis with Dunn's multiple comparison.

high-performance liquid chromatography (HPLC)-grade H₂O, which was sonicated for 2 min to increase dispersion of pigments in the aqueous solution and centrifuged at 4,000 rpm to correct for the splashing that occurred during sonication. To the suspension of pigment in H₂O, 30 μ l of 30% H₂O₂ and 375 μ l of 1 M K₂CO₃ were added. The vials were capped and secured on an orbital shaker set to ~200 rpm to digest at room temperature for about 20 h. After digestion, excess peroxide was destroyed with the addition of 50 μ l of 10% Na₂SO₃ and each sample was acidified with 140 μ l of 6 N HCl. These digested, quenched, and acidified samples were centrifuged at 13,000 rpm for 5 min to pelletize any undigested melanin, and then 300 μ l of supernatant was transferred into autosampler vials for chemical analysis. The LC-MS analyses were performed on an UltiMate 3000 UHPLC system coupled to an Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Pittsburgh, PA). Separations were performed on an Accucore HILIC LC column (150 $\times$ 2.1 mm; 2.6 μ m particle size, Thermo Scientific) kept at 25°C. For each analysis, 10 μ l of sample was injected onto the LC column and the chromatographic method employed used 0.1% formic acid in ACN and 0.1% formic acid in H₂O as mobile phases A and B. The chromatographic conditions were as follows: 0 min, 80% A; 10 min, 80% A; 10.1 min, 100% A; 20 min, 80% A; 25 min, 80% A; with a constant flow rate of 200 μ l/min. The high-resolution mass spectrometric (HRMS) experiments were all conducted in negative ion mode using an electrospray ionization (ESI) source. The ESI parameters used were a spray voltage of -4.0 kV, an aux gas flow rate of 8 units, sheath gas flow rate of 25 units, sweep gas set to 3 units, and capillary temperature set to 320°C. The mass spectrometer scanned a mass range of 120 to 1,800 m/z with a resolution of 140,000, an automatic gain control (AGC) target of 3.0×10^6 , and maximum injection time of 200 ms. The HRMS data were analyzed and processed using the MAVEN (Clasquin et al. 2012) software and processed to generate bar graphs (Supplementary Table S1). Extracted ion chromatograms were generated in MAVEN (Clasquin et al. 2012) with an extraction window of 5 ppm.

Seed sterilization and germination. For all seedling experiments, Col-0 accession *A. thaliana* plants were used. All seeds were surface sterilized in 70% ethanol with 0.1% Triton X-100 for 1 min, 10% household bleach with 0.1% Triton X-100 for 15 min, and three washes with sterile distilled water. Seeds were stratified for at least 3 days in the dark at 4°C and subsequently germinated at 24°C with 16 h of light for 6 to 8 days on agar plates containing half-strength (2.22 g/liter) Murashige & Skoog (MS) vitamins, 1% sucrose, and 1% Phytoagar (Bioworld).

Plant colonization experiments. To inoculate plates, 150 μ l of individual isolate resuspensions at OD 0.01 was spread on prepared quarter-strength MS square agar plates (150 mm $\times$ 150 mm) with no sucrose. For colonization experiments with SA, 100 μ l of isolate resuspensions was spread on quarter-strength MS square agar plates (100 mm $\times$ 100 mm) with no sucrose and 0.1 mM SA, which did not influence seedling survival after 14 days. Plates were allowed to dry and four to five seedlings were aseptically transferred onto each plate with flame-sterilized tweezers. Plates were sealed with Parafilm M Laboratory Film and randomly stacked vertically in open wire trays, which were grown at 24°C with 16 h of light for 14 to 15 days. Every 2 days, root length was observed, phenotype was assessed, and plates were shuffled. After 14 to 15 days, the seedlings from each plate were aseptically harvested and pooled in sterile previously weighed 1.5-ml centrifuge tubes. Tubes were weighed again after tissue was added to determine seedling biomass. To quantify internal levels of colonization, roots were rinsed and vortexed 5 s three times with sterile distilled water to determine combined level of colonization for internal and tightly attached

external bacteria. For homogenization of weighed seedlings, a combination of sterile garnet and 3 mm glass beads were aseptically transferred to tubes containing the pooled, surface sterilized whole seedlings, 1 ml of sterile phosphate buffered saline was then added to each tube, and samples were homogenized in a 2010 Geno/Grinder at 1,500 rpm for ~5 min. From homogenized tissue, 100 μ l was spread on LB plates and incubated at 28°C for 4 to 7 days. CFUs were counted and recorded. For experiments in pots, 64 ml of sterile calcined clay (Pro's Choice Rapid Dry) in three-inch square pots was inoculated with 49 ml normalized culture suspended in half strength MS buffered with sterile 2-(*N*-morpholino) ethanesulfonic acid (MES). Six-day-old seedlings were aseptically transferred to inoculated pots. An additional 1 ml of suspended inoculum was applied directly to seedling roots to bury them in the calcined clay. Plants were watered every 2 to 3 days from the top with sterile distilled water and grown in growth chambers (Percival, model AR41L3C8) with 10 h of light at 22°C and 14 h of dark at 18°C. Beginning at 6 weeks of growth, plants were aseptically harvested when inflorescence began to emerge. Whole plants were submerged in 25 ml of sterile harvesting phosphate buffer with 0.01% Silwet (Lehle Seeds) and vortexed vigorously for 10 s. Roots and rosettes were separated with sterile forceps and transferred to sterile centrifuge tubes and weighed to measure biomass (Supplementary Fig. S1).

Pangenomic visualization. Genomes for strains 303, OV320, 299, OV308, CL18, YR375, 136, OK210, and *Streptomyces scabiei* 87.22 (Supplementary Tables S2 and S3) were downloaded from Joint Genome Institute (JGI) Integrated Microbial Genomes & Microbiomes (IMG/M) expert review (ER) system (Chen et al. 2017). Genomes were mined using JGI IMG/M ER query tools and Anvi'o v2.1.0 (a platform used to analyze and visualize genomic data) (Eren et al. 2015). Pangenomic analysis was performed with Anvi'o, as outlined by Eren et al. (2015). Homologs were identified using similarity searches through NCBI BLASTP and protein clusters were resolved with the MCL algorithm (inflation parameter 6) (Enright et al. 2002) using the minbit scoring method (score 0.5). Annotations were done with clusters of orthologous groups (COG) (Galperin et al. 2014). Manual "binning" of protein clusters shared between subsets of genomes facilitated identification of group-specific gene clusters. Analyses resulted in a visual representation of the pangenome and a database of protein family annotations from the COG database (Galperin et al. 2014).

Phylogenetic trees and identification of *mel* operons. A concatenated alignment of the amino acid sequences of five housekeeping genes (*trpB*, *gyrB*, *rpoB*, *atpD*, and *recA*) from nine plant-associated *Streptomyces* strains and one *Kitasatospora* strain, which represents another genus in the family *Streptomycetaceae*, was used to build a maximum likelihood (RAXML v7.2.8) phylogenetic tree using 100 bootstrap replicates (Kearse et al. 2012). Alignment, using MAFFT v7.017 (Katoh et al. 2002), was performed in Geneious version R7 (Kearse et al. 2012) and tree building was done using RAXML-HP Black Box (version 8.2.10) through the CIPRES Science Gateway V. 3.3 (Miller et al. 2010). Melanin genes (*melC1* (SCAB85691), *melC2* (SCAB85681), *melD1* (SCAB59231), and *melD2* (SCAB59241)) from *S. scabiei* 87.22 identified by (Yang and Chen 2009) were used as queries for NCBI BLASTP searches to identify melanin genes in all strains (Yang and Chen 2009). Alignment (MAFFT v7.394) (Katoh et al. 2002) of amino acid sequences of the *melC2*, *melD2*, *melC1*, and *melD1* genes from six *Streptomyces* strains was used to build a Maximum Likelihood tree using RAXML-HP Black Box (version 8.2.10) through CIPRES Science Gateway V. 3.3 (for the tyrosinase tree) (Miller et al. 2010) or RAXML v7.2.8 through Geneious

version R7 (for cofactor tree). Housekeeping and *mel* trees were visualized in iTOL (Ciccarelli et al. 2006). Among the nine *Streptomyces* that we included in our analysis, six encoded at least one *mel* operon. All six strains encoded the *melD* genes, whereas only five strains encoded the *melC* genes (Fig. 2C and D).

Tyrosinase assay. Enzymatic oxidation of L-3,4-dihydroxy-phenylalanine (L-DOPA) by tyrosinase was monitored spectrophotometrically (Claus and Decker 2006) using the BioTek Synergy Multi-Detection Microplate Reader. Synthesis of Dopachrome was monitored at an absorbance of 475 nm. To prepare the assay, liquid cultures of each *Streptomyces* isolate were grown in 100 ml standard glucose-minimal salts medium with Tiger's Milk at 30°C with shaking for 5 to 7 days, according to Kieser et al. (2000). Approximately 20 ml of each culture was harvested and split between two 15-ml conical tubes, which were centrifuged for 7 min at 3,200 × *g*. Supernatants were collected and kept on ice while pellets were resuspended and washed with 10 ml of 0.1 M sodium phosphate (pH 6.8). Resuspended cells were then centrifuged for 7 min at 3,200 × *g* and supernatants were discarded. Following resuspension of cell pellets in 10 ml of 0.1 M sodium phosphate (pH 6.8), suspensions were moved to 100 µm silica bead tubes (Ops Diagnostics 100-100-01) and a 1× treatment of protease inhibitor cocktail (VWR M222-1ML) was added. Tubes were bead beaten for 10 min at 1,560 rpm and were treated with lysozyme using 40 µl of a 100 mg/ml stock and incubated for 30 min at 37°C. Cells were then centrifuged for 3 min at 13,000 × *g* and supernatants were collected, which was considered the cell extract fraction.

Supernatants were treated with 70% ammonium sulfate ((NH₄)₂SO₄) and incubated on ice until dissolved. After dissolution, extracellular protein extracts were centrifuged at 4°C for 30 min at 3,200 × *g*. Supernatants were discarded and protein pellets were resuspended in 2.5 ml of 0.1 M sodium phosphate. To remove the salt from the protein pellets, samples were applied to PD 10 desalting columns and allowed to pass through via gravity. Before application, columns were washed with four applications of 0.1 M sodium phosphate (pH 6.8). After desalting, total protein concentrations were determined via the microtiter Bio-Rad protein assay. Samples were normalized by protein concentration to 1,000 µg/ml and loaded into 96-well microtiter plates, which included the following: 100 µl of cell extract or extracellular extract all resuspended in 0.1 M sodium phosphate, 100 µl of 6.8 mM L-DOPA, and with or without 5 mM of a tyrosinase inhibitor kojic acid (KA) (Chen et al. 1991). For 299 supernatant protein extractions, 6 mM CuSO₄ was also added to the reaction to observe activity. KA is known to inhibit the enzymatic activity of tyrosinase (Chang 2009; Chen et al. 1991). KA has been shown to have a competitive inhibitory effect on monophenolase activity via copper chelation. Additionally, KA is known to have a mixed inhibitory effect on dephenolase activity of tyrosinase and likely inhibits tyrosinase via copper chelation at the enzyme's active site (Chang 2009). Controls included three replicates of wells without protein containing combinations of 0.1 M sodium phosphate, L-DOPA, and 5 mM KA. A tyrosinase standard was also run using lyophilized powder from Spectrum Chemical Group and 25,000 U (25 mg) of enzyme was diluted in 0.1 M sodium phosphate buffer (pH 6.8) and

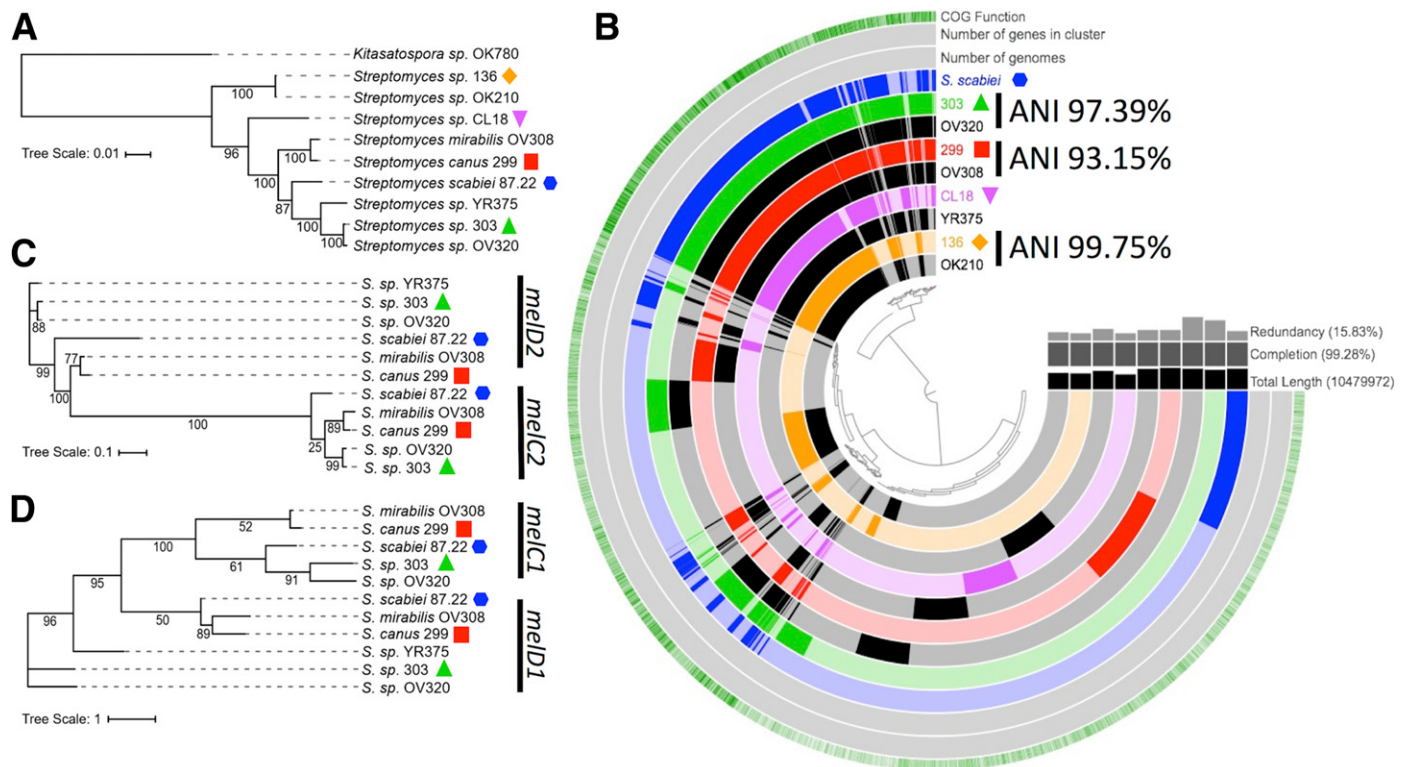


Fig. 2. Phylogenetic and pangenomic comparison of *Streptomyces* spp. indicate distinct phylogeny and overlapping genes consistent with melanin production. **A**, Phylogenetic tree built using a concatenated alignment of amino acid sequences for housekeeping genes *tpxB*, *gyrB*, *rpoB*, *atpD*, and *recA* from nine *Streptomyces* strains and one outgroup (*Kitasatospora* sp. OK780) (maximum likelihood, bootstrap consensus values based on 100 iterations). **B**, Pangenomic comparison of nine *Streptomyces* isolates, including 136 (orange), CL18 (purple), 299 (red), 303 (green), and *S. scabiei* (blue). Maximum likelihood trees built using amino acid alignments of **C**, tyrosinase genes and **D**, tyrosinase helper genes from six pigment-producing *Streptomyces* reveal distinct clades that separate with a *S. scabiei* single annotated gene (*melC1*, *melC2*, *melD1*, and *melD2*) falling into each clade (bootstrap consensus values based on 100 iterations).

subsequent standards were prepared in a range from 1 to 15 activity units for reads (Supplementary Fig. S2). Absorbance at 475 nm was measured every minute for 3 to 11 h.

Phenolic compound challenges on agar. To determine strain resistance to phenolic compounds, standard glucose-minimal salts medium (MM) was prepared with the addition of 0.01 g of CuSO₄ and 100 ml of phosphate buffer (NaH₂PO₄ at 138 g/liter and Na₂HPO₄ at 142 g/liter) per liter. Filter sterilized phenolic compounds were added to cooled, autoclaved media at concentrations of either 0, 0.125, 0.5, 1, or 5 mM for salicylate (salicylic acid, SA), catechol, and ferulate (ferulic acid, FA). Twenty-five milliliters of phenol-containing agar was pipetted or poured onto petri dishes. Once solid, 100 µl of *Streptomyces* strains 299, 303, CL18, and 136 standardized to OD₆₀₀ of 0.01 (described previously) and diluted 1:10 was pipetted onto solidified plates and spread with sterile beads (CFU range: 4×10^3 to 4×10^4). Plates were incubated at 30°C and checked daily for CFU formation. CFUs were counted after 4 to 7 days of incubation and recorded.

SA challenge in liquid media. Liquid cultures of each isolate were first prepared as described above with the exception of growth medium type. Cultures were grown in liquid MM (Kieser et al. 2000) with CuSO₄ rather than solid LB. A spectrophotometer measured the optical density at 600 nm (OD₆₀₀) and cultures were normalized to an OD₆₀₀ of 0.1. To inoculate flasks, 100 µl of all normalized isolate resuspensions was added to sterile 125-ml flasks containing 75 ml of MM and concentrations of either 0.0 or 0.5 mM SA. These concentrations were chosen based on results from solid media as well as previous findings from Lebeis et al. (2015). In addition, a separate complete set of flasks was inoculated exactly as described, with the addition of 1.5 mM KA. Three replicate cultures were prepared. Erlenmeyer flasks were incubated at 30°C with shaking at 125 rpm for 6 days. After the 6 day incubation, biomass was collected via 10 s vacuum filtration of each 75-ml culture on 0.2 µm filter paper. Filters were allowed to dry overnight. Three filter paper controls with filtered liquid media only were weighed, and biomass for each sample was calculated based on the average mass of the control filter paper subtracted from the filter paper with *Streptomyces* biomass.

303 tyrosinase expression in seedlings. Axenic, Col-0 6- to 7-day-old seedlings were left sterile or inoculated with 303 as described above previously and grown for 10 days. At 10 days, individual plants were placed in bead tubes containing 100 µm silica, 4 mm silica, and 1.7 mm zirconium beads (Ops Diagnostics 4000-100-28). For positive controls, in vitro 25 ml of 303 cultures grown in LB for 6 days were spun down and placed in bead tubes containing 100 µm silica beads (Ops Diagnostics 100-100-02). One milliliter of TRIzol reagent was added to each tube, and samples were homogenized in a 2010 Geno/Grinder for 10 min at 1,560 rpm. After homogenization, tubes were centrifuged at 12,000 × g for 5 min and the supernatant was transferred to a new microcentrifuge tube. To each tube, 200 µl of Chloroform was added, and samples were incubated for 15 min at 4°C. Phase separation was performed by centrifugation at 12,000 × g for 5 min at 4°C. The upper aqueous phase was removed, and the RNA was cleaned using the RNA Clean and Concentrator kit for TRIzol clean-up (Zymo R1015). RNA was converted to cDNA using Quantabio qScript (95048-025) and a PCR was performed using 303 *melC* specific primers to amplify a 665-bp range inside the *melC* operon using the SnapGene program. Strain 303MelCFwd primer (CAGTCGGTGTGCAAGGT GTAGTG) and 303MelCRev_01205 (CACCGTTCCTTCCTTCC TGC) were used. PCR conditions were 3 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 64°C, and 1 min at 72°C followed by 5 min at 72°C. Following the PCR, a 1% agarose gel electrophoresis was performed to verify that a 665-bp product was specifically amplified.

Statistical analysis. LC-MS ion counts, seedling colonization, phenolic compound challenge, culture biomass, and plant biomass results were statistically analyzed with Prism version 7.0a for Mac (GraphPad Software, La Jolla, CA, <https://www.graphpad.com>). Two-way ANOVA with Dunnett's multiple comparisons were used for LC-MS peak ion counts. For seedling colonization, culture biomass, and plant biomass results, a Kruskal-Wallis with Dunn's multiple comparisons was used. For phenolic compound challenge, a two-way ANOVA with a Fisher's LSD was used to determine differences between strains at each phenolic compound concentration.

RESULTS

Melanin production is associated with root-enriched *Streptomyces* isolates. When *A. thaliana* was inoculated with a defined bacterial community of isolates and grown for 6 to 8 weeks, two *Streptomyces* isolates, 299 and 303, displayed enriched root endophytic compartment colonization while another two, CL18 and 136, did not (Lebeis et al. 2015). Strikingly, 299 and 303 cultures of liquid and solid media developed distinct pigmentation in vitro, which never appear in CL18 or 136 cultures (Fig. 1A). Pigment produced by strain 299 was delayed and lighter in color than that produced by 303 (Fig. 1A). The brown/black color suggested the potential production of a melanin pigment.

Pigments produced by cultures of 303, 299, and CL18 was extracted from spent liquid media. No pigment pellet resulted from extraction performed on spent 136 liquid cultures. LC-MS analysis of pigments extracted from 299 and 303 indicated molecular similarity to a synthetic melanin standard by the presence of two distinct melanin degradation products (pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA) (Ito and Wakamatsu 1998), while the CL18 samples were not significantly different than the blank sample controls (Fig. 1B). Overall, our findings suggest that 299 and 303 produce an extracellular melanin in vitro, while CL18 and 136 do not.

Seedling colonization is *Streptomyces* strain-specific. In a mixed bacterial community, our two melanin-producing strains 299 and 303 were significantly more abundant in mature roots than in inoculum, while our nonproducing strains CL18 and 136 were not (Lebeis et al. 2015). Thus, to determine if colonization differences are observed earlier and distinguish influences of mixed bacterial communities from individual strain capabilities, *Streptomyces* isolates 299, 303, CL18, and 136 were screened for their ability to colonize *A. thaliana* seedlings as the sole inoculum for 14 days (Fig. 1C). After 2 weeks, seedlings were colonized with significantly higher concentrations of isolates 299 and 303 than 136, whereas CL18 colonization was not significantly different from any other isolate in this mono-association (Fig. 1C). Importantly, plants growing in mono-association with these strains show no signs of pathology or change in biomass even after 6 to 8 weeks of growth with *Streptomyces* colonization, confirming that they are not pathogens or growth promoting under these conditions. Thus, in addition to increased root colonization of mature plants when competing with other bacteria (Lebeis et al. 2015), 299 and 303 colonize seedlings better than 136, while CL18 is not significantly different from the other *Streptomyces* (Fig. 1C).

Genomes of melanin-producing strains contain genes essential for melanin production. To reveal genetic similarities among *Streptomyces* strains with higher plant colonization, we decided to compare the genomes of 303, 299, CL18, and 136 with a selection plant-associated *Streptomyces* isolates. For this analysis, we added four additional *Streptomyces* strains isolated from Poplar trees. We also included the plant pathogen *S. scabiei* 87.22 in this

comparison for its robust colonization of *A. thaliana* roots and melanin pigment production. *S. scabiei* 87.22 proved more closely related to 299 and 303 than CL18 or 136 (Fig. 2A), although none of our strains are pathogens. Besides the genome of *S. scabiei* 87.22, which is complete, all genomes were estimated to be >99% complete by Anvi'o identification of four sets of bacterial single-copy gene collections (Eren et al. 2015). Our genome comparison revealed that 303, 299, and *S. scabiei* 87.22 had larger genomes with 1,500 to 3,000 additional genes than CL18 and 136, leaving room for exploration of genes and potential gene products involved in plant association (Fig. 2B). Among the four additional plant-isolated *Streptomyces* from Poplar trees, three had remarkably high genome identity (>93% ANI) with 303, 299, or 136 (Fig. 2B, Supplementary Table S4). We next performed a pangenomic analysis of the nine *Streptomyces* strains to investigate conserved and unique genes across their whole genomes. Among genes present in 303, 299, and *S. scabiei*, but absent in CL18 and 136 (Supplementary Table S5), were those responsible for melanin production, providing us with the opportunity to explore a potential link between the ability of microbes to survive oxidative stress and colonize plants.

BLASTP searches for genes encoding tyrosinase enzymes, which are known to be required for *Streptomyces* melanin production, returned two copies in the genomes of 303, OV320, 299, OV308, and *S. scabiei*, and a single copy in the genome of YR375 (Fig. 2C). Directly, upstream of each tyrosinase is its annotated cofactor encoding the helper protein (Fig. 2D) responsible for copper ion addition to tyrosinase allowing enzymatic function (Yang and Chen 2009). Because we found multiple copies of the genes encoding tyrosinases and their cofactors, we searched for the presence of distinct *melC* and *melD* operons in our select *Streptomyces* genomes to suggest potential distinguishable extracellular and intracellular tyrosinases (Yang and Chen 2009). Phylogenetic trees of tyrosinase genes and helper genes cluster into two groups with a single represented *mel* homolog gene from *S. scabiei* present in each (Fig. 2C and D). Further, the predicted *melC1* and *melC2* genes were contiguous within each genome, which was also true for the predicted *melD1* and *melD2* genes. Components of the melanin operon were not identified in the genomes of CL18 or 136. Therefore, our genome comparisons identify two distinct *mel* operons in the genomes of melanin-producing strains 303 and 299.

Enzyme kinetics differentiate tyrosinase activity between *Streptomyces* strains. While extracellular melanin production was observed in 303 and 299 in vitro cultures, it was unclear what the potential activity of a second tyrosinase/cofactor pair might confer. We next sought to determine if 299 and 303 did indeed produce both functional intracellular and extracellular tyrosinases by performing an enzymatic activity assay on whole cell extracts and culture supernatants of all four isolates. Whole cell (intracellular) and supernatant (extracellular) protein extracts from all four strains were combined with tyrosinase substrate L-DOPA, and enzymatic activities were observed by dopachrome production. While strain 303 showed enzymatic activity in the extracellular and intracellular protein extracts, the enzymatic activity in the whole cell protein extract was slower (Fig. 3A). Interestingly, the tyrosinase activity demonstrated in 303 intracellular and extracellular protein extract could be inhibited by the copper chelation activity of KA in vitro (Fig. 3A). Extracellular 299 tyrosinase activity required exogenous CuSO₄ to observe activity and was slower compared with 303 extracellular tyrosinase (Fig. 3B), corresponding to delayed pigment production in vitro (Fig. 1A). Intracellular 299 tyrosinase activity was similar in dynamics to intracellular 303 and extracellular 299 tyrosinases (Fig. 3B). Finally, intracellular and extracellular tyrosinase activity in 299 could not be inhibited with KA

(Fig. 3B). Under similar conditions, we did not detect an enzymatic activity in CL18 or 136 cultures. Together, our results suggest that while 299 and 303 both produce melanin, the tyrosinase required for its production has different activity and susceptibility to tyrosinase inhibitors in the two strains (Fig. 3).

Survival and growth in the presence of phenolic compounds is improved in enriched colonizers. Microorganisms living close to and within plant roots must contend with root exudates, potentially including phenolic compounds such as SA and FA, which inhibit *Streptomyces* growth (Yang and Chen 2009). SA is measurable in *A. thaliana* roots and seedlings while both SA and FA are present in root exudates of *A. thaliana* and *Avena barbata* (Chaparro et al. 2013; Lebeis et al. 2015; Zhalnina et al. 2018). To determine if melanin-producing *Streptomyces* isolates have greater tolerance to these phenolic compounds, we counted surviving colonies of all isolates on solid MM impregnated with varying concentrations of catechol, SA, and FA (Fig. 4). As seen in Figure 4A, catechol challenge resulted in significantly more colonies for 303 and 299 than CL18 and 136 at 0.125 and 0.25 mM concentrations. When challenged with FA, a lignin degradation product (Kirby 2006) at concentrations of 0.125, 0.5, and 1 mM, 303 grew significantly better than all other strains (Fig. 4B). At concentrations of 0.125, 0.25, and 0.5 mM SA, 303 grew significantly better than 299, CL18, and 136 (Fig. 4C). Therefore, *Streptomyces* isolates with two functional copies of tyrosinase are more resistant against phenolic compounds than isolates with no tyrosinases.

Isolate 303 also encodes enzymes in a *Streptomyces* SA degradation pathway while similar genes were not found in the genomes of 299, CL18, and 136 (Lebeis et al. 2015). To determine if the increased resistance to SA on solid medium was due to increased growth of 303, or increased protection from SA, we added the tyrosinase inhibitor KA liquid minimal medium with SA and measured biomass accumulation after 6 days of growth. Biomass comparisons of 303 indicated that SA addition does not increase growth (Fig. 4D), suggesting that 303 does not have increased growth under these in vitro conditions, but rather is protected from SA.

Influence of SA and tyrosinase on seedling colonization. Our in vitro assays demonstrate that SA directly prevents *Streptomyces* colony formation (Fig. 4A to C). To determine if SA negatively influences *Streptomyces* isolate seedling colonization, 0.1 mM SA was added to seedlings during a 14-day colonization experiment. Isolates 303 and 299, which have functional tyrosinases, were still able to significantly colonize seedlings (Fig. 5A). However, CL18, which does not have a functional tyrosinase, did not colonize significantly higher than the no bacteria control (Fig. 5A). Interestingly, 136, which also lacks a functional tyrosinase, no longer colonizes at significantly lower levels than 303 and 299. To establish that *Streptomyces* tyrosinase was expressed, and could therefore potentially protect microbes from SA during colonization, we performed RT-PCR on *A. thaliana* seedlings 10 days after inoculation with 303 using 303 *melC2* specific primers. Thus, we were able to demonstrate expression in 303 inoculated seedlings, which could not be detected in uninoculated seedlings controls (Supplementary Fig. S3). Together, we see that when SA is applied to seedlings during colonization, 303 and 299 with their functional *MelC* and *MelD* maintain their colonization patterns, while CL18 and 136 do not.

In genome comparison of nine selected *Streptomyces* isolates, we determined that several isolates from Poplar trees in Oregon shared a high degree of identity with our strains (Fig. 2B). We found that OV320 and OV308 also produced melanin in vitro, had two identified copies of tyrosinase in their genomes, and exhibited similar intracellular tyrosinase activity to 303 and 299 (Supplementary Fig. S4). OK210 did not produce melanin in vitro and did not

encode any tyrosinase in its genome. When *A. thaliana* seedlings were grown with these isolates for 14 days, we observed that OV320 and OV308 colonized significantly better than OK210 (Fig. 5B), as predicted from their closely related strains 303, 299, and 136 (Fig. 2B). The level of colonization when SA was added to these mono-associations with seedlings was also similar to their close relatives with OV320 and OV308 maintaining significant colonization, despite SA presence. Further, as with 136, OK210 is not significantly different from OV320 and OV308 under these stressful colonization conditions (Fig. 5C). Finally, we also colonized seedlings with strain YR375, which was isolated from a

Poplar tree in North Carolina. This strain sporadically made melanin in vitro and only encoded a single tyrosinase gene, which fell in the same clade as *melD* (Fig. 2C). Because *melD* is the predicted intracellular copy of tyrosinase in *S. scabiei* (Yang and Chen 2009), we suspect the sporadic melanin production observed occurred when cells lysed during in vitro growth. We observed sporadic colonization with this isolate, which was significantly lower than OV320 and OV308, but not significantly different from the no bacteria control or OK210 (Fig. 5B). When we investigated gene clusters shared within the genomes of 303, OV320, 299, OV308, and *S. scabiei*, but not YR375, CL18, 136, or OK210, we observed

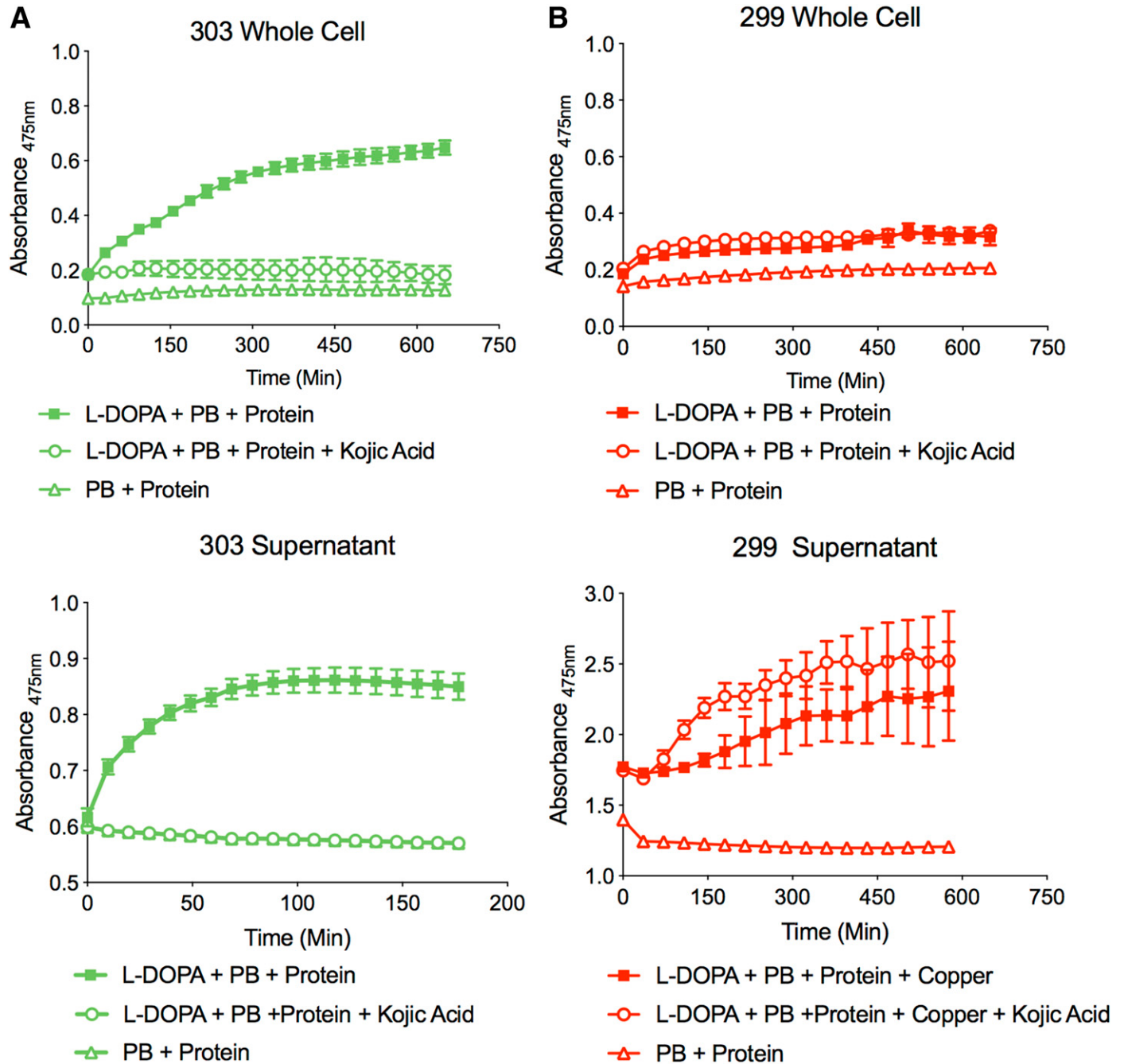


Fig. 3. Strain-specific tyrosinase activity in intracellular and extracellular protein extracts from 303 and 299 in vitro cultures. Dopachrome production was measured by absorbance at 475 nm for three replicate cell pellet (top panels) and supernatant (bottom panels) protein extracts from **A**, 303 and **B**, 299, which were normalized by protein concentration following the addition of phosphate buffer alone (open triangles), phosphate buffer and L-DOPA (closed squares), or phosphate buffer, L-DOPA, and kojic acid (open circles). For 299 supernatant, 6 mM CuSO_4 was added to observe activity. The mean and standard deviation is displayed for each graph.

that the second copy of the *mel* operon, which encodes the extracellular MelC tyrosinase was exclusively shared by those strains with significantly higher colonization of *A. thaliana* seedlings, even when SA was present.

DISCUSSION

Streptomyces are capable of colonizing the roots of a wide variety of plant species (Bonaldi et al. 2015; Bulgarelli et al. 2012; Edwards

et al. 2015; Lebeis et al. 2015; Lundberg et al. 2012; Peiffer et al. 2013; Yeoh et al. 2016) in geographically and geologically diverse soils (Choudoir et al. 2016), emphasizing the need to understand their assembly into the root microbiome. Select *Streptomyces* strains have been identified as plant growth promoting and even disease suppressive (Viaene et al. 2016), highlighting their potential applications in agriculture. Although microbial activities associated with root colonization for more predominant taxa, including Proteobacteria, have been elucidated, specific functions involved in

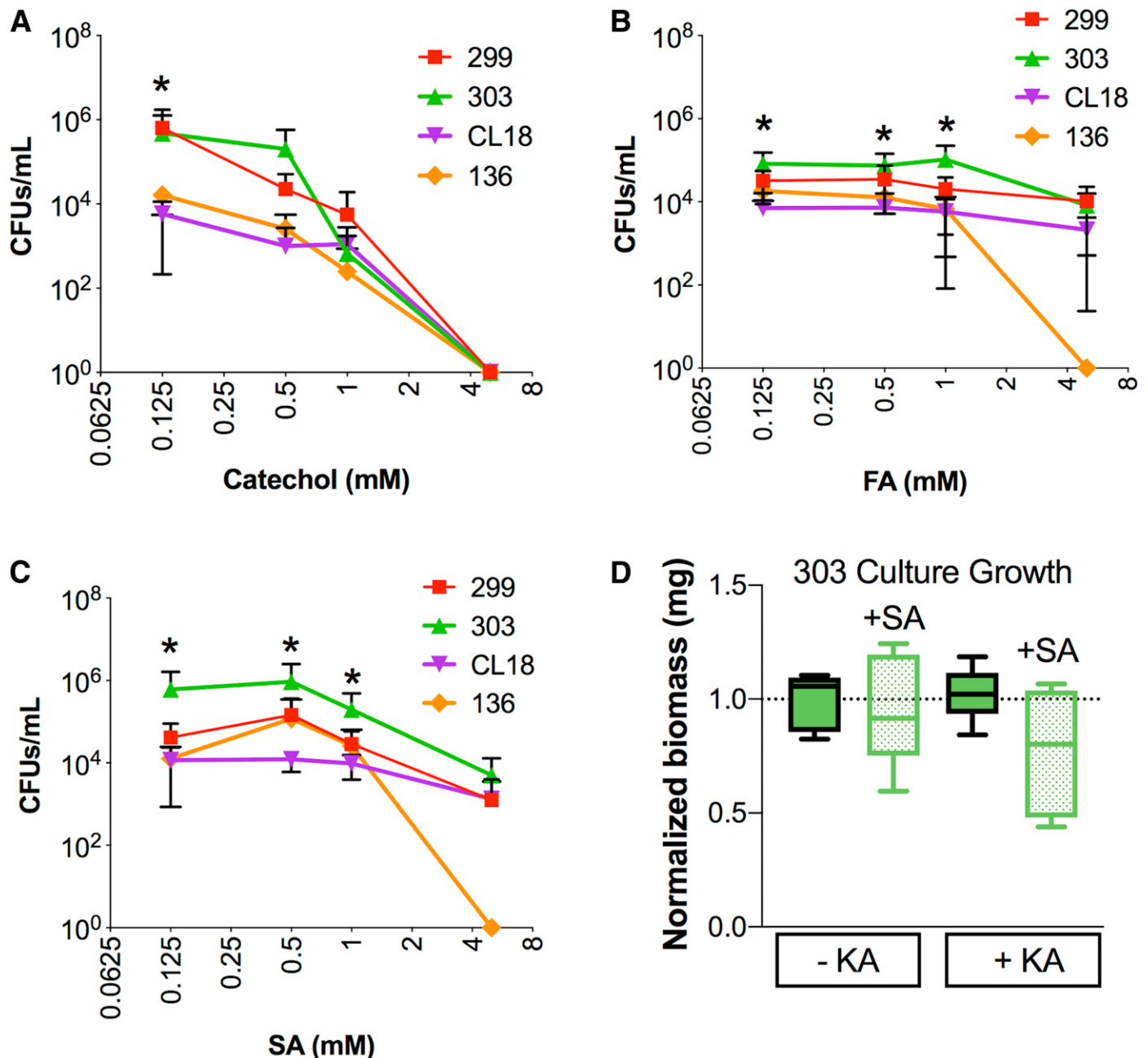


Fig. 4. Phenolic compound tolerance differs between *Streptomyces* isolate. Strains (303, green; 299, red; CL18, purple; and 136, orange) were grown on solid minimal media containing the indicated concentrations of **A**, catechol, **B**, ferulic acid (FA), or **C**, salicylic acid (SA). In **A**, * indicates 303 and 299 are significantly greater than CL18 and 136 according to two-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) ($n = 6$ to 9 , $P < 0.05$). In **B**, * indicates 303 is significantly higher than 299, CL18, and 136 according to two-way ANOVA and Fisher's LSD, ($n = 6$ to 9 , $P < 0.05$). In **C**, * indicates 303 is greater than 299, CL18, and 136 according to two-way ANOVA and Fisher's LSD ($n = 6$ to 9 , $P < 0.05$). **D**, Biomass of 75 ml of liquid culture grown for 6 days in buffered minimal medium with (patterned green) and without (solid green) kojic acid (KA) with and without the addition of 0.5 mM SA. The average biomass of the no KA, no SA control was used to normalize the biomass values between experiments, which was run twice in triplicate, with no significant differences found, using a Kruskal-Wallis with Dunn's multiple comparison.

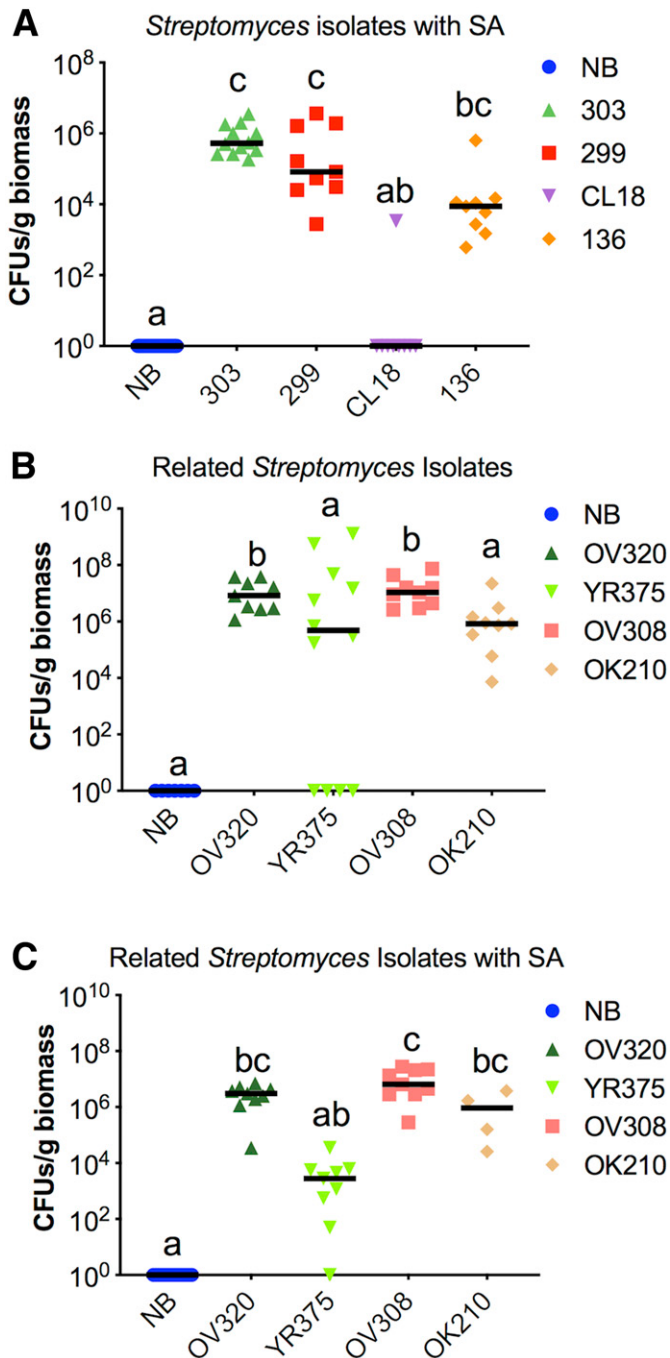


Fig. 5. Differential colonization of *Arabidopsis thaliana* seedlings by *Streptomyces* isolates with the application of salicylic acid (SA). **A**, Axenic 6- to 7-day-old Col-0 wild-type seedlings were singly inoculated with each *Streptomyces* isolate (no bacteria control [NB, blue circles], 303 [green triangles], 299 [red squares], CL18 [purple triangles], 136 [orange diamonds], $n = 9$ to 12) and grown with 0.1 mM SA. After 14 days, plants were rinsed and seedling colonization level was determined. Different letters indicate statistical differences using a Kruskal-Wallis with Dunn's multiple comparison. **B and C**, Axenic 6- to 7-day-old Col-0 wild-type seedlings were singly inoculated with four plant-associated *Streptomyces* isolates with genetic similarity to 303, 299, and 136 (no bacteria control [NB, blue circles], OV320 [dark green triangles], YR375 [light green triangles], OV308 [light red squares], and OK210 [dark orange diamonds], $n = 4$ to 12) and grown for 14 days **B**, without SA or **C**, with 0.1 mM SA when rinsed seedling colonization level was determined. Different letters with B and C indicate statistical differences using a Kruskal-Wallis with Dunn's multiple comparison.

root microbiome assembly mechanisms for less abundant microbes, such as *Streptomyces* are largely unexplored (Fitzpatrick et al. 2018; Jones et al. 2017; Viaene et al. 2016; Yandigeri et al. 2012). Here we begin to investigate a set of plant-associated *Streptomyces* isolates and identify genes shared by *Streptomyces* that may influence early stages of colonization in seedlings.

Based on our ability to distinguish *Streptomyces* strains by degree of root and seedling colonization, we hypothesized genomic differences would explain strain variation. Our comparative genomic analyses suggest distinct differences between *Streptomyces* isolates. These findings highlight that functional conclusions based on genus-level abundance from 16S rRNA gene amplicon studies inadequately capture the organisms' potential, as previously described for plant-associated *Pseudomonas* strains (Blakney and Patten 2011). Further, including the plant pathogen *S. scabiei* 87.22 in our genomic comparisons revealed shared genetic factors contributing to increased seedling colonization in our nonpathogenic, melanin-producing *Streptomyces* isolates. Interestingly, Beausejour and Beaulieu (2004) found that *S. scabies* virulence and colonization was reduced in mutants that lost the ability to make melanin. Beyond their role in melanin production, the tyrosinase enzymes encoded by *Streptomyces* are capable of oxidizing various phenolic compounds, including SA and FA, into their quinone form (Yang and Chen 2009). The data we present here using phenolic compounds previously shown to induce ROS production (Bosund et al. 1960; Cheng et al. 2013) suggest that plant-associated strains living on or near roots with multiple tyrosinases may better resist phenolic root exudates during colonization.

Although SA is not assimilated by *Streptomyces* during *A. thaliana* colonization (Worsley et al. 2019), it does influence *Streptomyces* assembly into a mature root microbiome through the immune responses or direct antimicrobial activity (Lebeis et al. 2015). In SA-containing inoculated liquid medium, KA did not significantly influence 303 biomass accumulation, suggesting that there are other mechanisms to alleviate SA-induced stress, such as the previously identified SA degradation pathway in 303 (Lebeis et al. 2015) or other tyrosinase products. Taken together, our genomic, in vitro, and in vivo findings emphasize that 299 and 303 tyrosinases have differential enzyme coding sequences, substrates, and products, supporting strain-specific SA-induced oxidative stress survival adaptations. A critical next step is defining the role of melanin and/or MelC tyrosinase is the generation of a targeted mutant strain. Further, such phenolic compounds are likely just one of many potential selective pressures imposed by the plant host.

Previous studies established that genomes of plant-associated bacteria were larger than the genomes of non-plant-associated bacteria (Levy et al. 2018), which might contain a number of genes to aid in the transition of living in bulk soil to within plant tissue. Likewise, we found that the *Streptomyces* isolates that colonize plants better have larger genomes coding for a wide range of potential functions. We chose to focus on *mel* genes based on a clear in vitro phenotype, widespread occurrence in environmental *Streptomyces* isolates, and the potential links to mitigating oxidative stress. Our data suggest that during plant colonization, melanin-producing strains are protected against phenolic compounds commonly found in the root-soil interface during colonization (Bakker et al. 2012; Chaparro et al. 2013; Hartmann et al. 2009; Kirby 2006; Strehmel et al. 2014; Zhelnina et al. 2018). Although our four additional plant-associated *Streptomyces* strains (OV320, OV308, YR375, and OK210) were isolated from trees in Oregon and North Carolina, their colonization patterns of *A. thaliana* seedlings correlate with their number of tyrosinase gene copies with significantly higher colonization in those isolates that contain *melC*, not just *melD*. Previous studies noted that *melD* was more common

than *melC* among environmental *Streptomyces* (Yang and Chen 2009). The impact of this finding is particularly interesting given that melanins are produced by a range of other soil-isolated fungi and bacteria (Drewnowska et al. 2015; Eisenman and Casadevall 2012; Plonka and Grabacka 2006). Understanding this new context for melanin production advances our understanding of the complex process of root microbiome assembly and manipulation.

As we seek new strategies to tackle the challenges of climate change-induced crop decline, we look toward the potential of the plant microbiome. Recently, plant drought resistance bacterial community studies identified *Actinobacteria* and more specifically *Streptomyces* as root enriched in drought conditions (Fitzpatrick et al. 2018; Xu et al. 2018). In order to more fully understand *Streptomyces* microbiome-associated capabilities, additional strain-level genomic and in situ studies are essential. Thus, we suggest the power of linking taxonomic identification and abundances to distinct strain gene comparisons and product exploration. Together our findings provide opportunities for harnessing the power of *Streptomyces* to improve plant health and more broadly enhance agricultural applications and crop productivity.

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