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Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa

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Immune systems distinguish "self" from "non-self" to maintain homeostasis and must differentially gate access to allow colonization by potentially beneficial, non-pathogenic microbes. Plant roots grow within extremely diverse soil microbial communities, but assemble a taxonomically limited root-associated microbiome. We grew isogenic *Arabidopsis thaliana* mutants with altered immune systems in a wild soil and also in recolonization experiments with a synthetic bacterial community. We established that biosynthesis of, and signaling dependent on, the foliar defense phytohormone salicylic acid is required to assemble a normal root microbiome. Salicylic acid modulates colonization of the root by specific bacterial families. Thus, plant immune signaling drives selection from the available microbial communities to sculpt the root microbiome.

Recognition of plant pathogens in leaves leads to dramatic changes in transcription, synthesis of defense phytohormones and antimicrobial compounds, and elaboration of physical barriers (*1*, *2*). Defense phytohormones are structurally-diverse plant secondary metabolites that integrate plant immune system output responses while repressing cell growth and proliferation. Salicylic acid (SA), jasmonic acid, and gaseous ethylene mediate localized and systemic plant immune responses (*3*, *4*). Non-specific systemic acquired resistance is mediated by SA in leaves (*5*). By contrast, induced systemic resistance in leaves can be triggered by specific rhizobacteria colonizing roots, and is mediated by jasmonic acid and ethylene (*4*). SA and jasmonic acid act antagonistically in responses to infection by biotrophs, at least in leaves (*6*). The defense phytohormones control a set of overlapping signaling sectors, each contributing to regulation of plant defense via transcriptional and biosynthetic output in leaves (*7*).

Accessions of *A. thaliana* show variation in defense phytohormone profiles after infection, even though they share similar root-associated bacterial microbiota (*8–10*). Previous studies examined the roles of defense phytohormones in shaping the wildtype root microbiome using single mutant lines defective in their biosynthesis or perception, or exogenous defense hormone application in combination with bacterial culturing and/or lower resolution profiling methods. No generalizable clarity has emerged to date (*11*, *12*). We therefore compared the bacterial root microbiome of wildtype *A. thaliana* accession Col-0 with a set of isogenic mutants lacking biosynthesis of, and/or signaling dependent on, at least one of the following: SA, jasmonic acid, and ethylene. We focused on multimutants that eliminated overlapping defense signaling sectors (Fig. 1A and table S1) (*13*). We anticipated that this experimental design would reveal the contributions of plant defense phytohormones to wildtype root microbiome composition.

We profiled bacterial communities of rhizosphere (soil directly adjacent to the root) and endophytic compartment (EC) from roots grown in a previously characterized wild soil from the UNC Mason Farm biological preserve, as well as unplanted bulk soil (figs. S1 to S4; tables S2 to S4; Methods 1-3, 6a-d) (*10*). Sample fraction (soil, rhizosphere, or endophytic compartment) and the differentiation of endophytic samples from bulk soil and rhizosphere explained the largest portions of variance across the bacterial communities examined (table S5) (*8*, *10*). Endophytic bacterial communities were less diverse than bulk soil and rhizosphere communities (Fig. 1B, fig. S4) with reduced representation of Acidobacteria, Bacteroidetes, and Verrucomicrobia, and enrichment of Actinobacteria and Firmicutes (ANOVA, q-value < 0.05). Individual Proteobacteria families were either enriched or depleted in endophytic

communities compared to soil and rhizosphere samples (fig. S5; Method 6b). These results are consistent with distributions of bacterial phyla from *A. thaliana* roots grown in four wild soils (*8*, *10*).

Plant genotype affected phylum-level bacterial root endophytic community composition (4.3-5.0%, Canonical Analysis of Principal Coordinates [CAP; (*14*); Fig. 1B; Method 4b and 6e] with both hyperimmune *cpr5* and immunocompromised quadruple *dde1 ein2 pad4 sid2* mutant communities displaying lower alpha-diversity indices than wildtype (Fig. 1B, fig. S4B; Methods 1b). The relative abundance of Firmicutes was lower in immunocompromised *jar1 ein2 npr1*, *ein2 npr1*, and *npr1 jar1* mutants, which all lack response to SA (Fig. 1, A and B, and table S1). Actinobacteria were less abundant in *cpr5* and *pad4* endophytic samples, whereas Proteobacteria were more abundant in *cpr5* and *jar1 ein2 npr1* (Fig. 1, A and B; fig. S8; Methods 4a). Only mutants that lacked all three defense hormone signaling systems exhibited diminished survival that correlated with the presence of an unidentified oomycete in the root microbiota of survivors (fig. S2; Methods 3 g).

We identified bacterial families and operational taxonomic units (OTUs) in the root endophyte microbiome of each mutant plant line that were differentially-abundant compared to wildtype plants using a Zero Inflated Negative Binomial (ZINB) model (tables S6 and S7; fig. S9; Method 6b). Both the number of differentially-abundant bacterial taxa and their identity differed in endophytic samples from mutants. Among 52 differentially-abundant families in surviving *dde1 ein2 pad4 sid2* mutant endophytic samples, nearly all were depletions (Fig. 1C and fig. S6), consistent with this mutant's decreased alpha-diversity (Fig. 1B). Differentially-abundant bacterial families are consistent with the significant relative phyla differences observed in specific defense hormone mutants (Fig. 1B and fig. S6A). In *cpr5*, for example, nine Actinobacteria families were identified with decreased relative abundance and 12 Proteobacteria families were identified with increased relative abundance, in comparison to wildtype (Fig. 1C, fig. S5, and table S6). These differences demonstrate that defense phytohormones modulate root microbiome composition at multiple taxonomic levels from phylum to family.

We then compared the enrichment and depletion profiles across the mutant genotypes to identify shared patterns (fig. S6C; Method 6d). Two striking genotype groups were observed. (Fig. 1C). Group 1 mutants constitutively produce and accumulate SA while group 2 mutants either accumulate less, or cannot respond to it. These two genotype groups exhibited complementary patterns of differentially-abundant Proteobacteria: in group 1 these were α-and β-Proteobacteria, while in group 2 they were γ-Proteobacteria (table S6 and fig. S6A). Within genotype group 2, nearly all of the differentially-abundant bacterial families in *sid2* were shared with *pad4* and *dde1 ein2 pad4 sid2*, especially those families depleted compared to wildtype, while half of the *dde1 ein2 pad4 sid2* depletions were apparently SA-independent (Fig. 1D and fig. S6B).

We re-analyzed the data to ask whether the differential family abundances observed in specific mutant groups remained consistent at lower taxonomic (OTU) resolution (table S4, tab B; table S7; Methods 6b). We largely recapitulated mutant groups 1 and 2 at OTU resolution (fig. S7B). If the plant selected bacteria at a low (genus or species) taxonomic level, we would expect that only one or a few abundant OTUs would drive, and thus correlate with, family-level analyses. However, we observed that a number of OTUs from across the abundance range matched family-level enrichment profiles (fig. S7C-F; Methods 6b). Importantly, these results suggest that defense phytohormones, particularly SA, modulate taxonomic groups of bacteria at the family level in the root, and not by altering the abundance of a small number of dominant strains within each differentially abundant family.

We next asked whether the bacterial families affected by the plant defense phytohormone mutants corresponded to taxa that were normally either enriched or depleted in wildtype roots compared to soil. We re-sequenced two regions of the 16S gene for a subset of the samples using a different technology. This allowed us to eliminate sequencing and amplification biases. We identified 19 enriched and 23 depleted families in endophytic samples of wildtype roots compared to soil (table S8; fig. S11; Method 6c). Consistent with phyla level analyses (Fig. 1B), 79% of the bacterial families enriched in endophytic samples were Actinobacteria or Proteobacteria. Further, 55% of the endophytic-enriched families in SA mutants are Actinobacteria or Proteobacteria (table S6, S8). A similar pattern was observed in the OTU level analysis, where 42% and 48% of the endophytic-enriched bacterial families contained at least one OTU that is further enriched in the phytohormone mutants (table S7, S8).

Six of the 19 endophytic-enriched families (table S8) were *depleted* in the *cpr5* mutant that constitutively produces SA (table S6), suggesting that these six bacterial families are sensitive to SA or SA-dependent processes. Five different endophytic-enriched families (table S8) were *further enriched* in group 2 mutants that lack SA-biosynthesis and signaling (table S6). Thus, these five bacterial families are candidates for taxa whose colonization is normally limited by wildtype levels of SA and/or SA-dependent processes. In contrast, 12 of the 23 endophytic-depleted families (table S8) were *further depleted* in group 2 mutants, but not in group 1 mutants. Hence, these endophytic-depleted families may require SA-dependent processes to maintain even their very low abundance in the wildtype endophytic compartment (tables S6 and S8). Thus, SA is required to modulate the assembly of a normal root microbiome. In its absence, core root bacterial community composition is significantly altered. However, these changes to the bacterial microbiome are not sufficient to alter survival of these mutants in this

particular wild soil.

We asked whether bacteria isolated from roots can colonize sterile roots in the context of a defined but complex synthetic bacterial community. We germinated surface-sterilized seeds (wildtype and defense phytohormone mutants) on a calcined clay substrate inoculated with a synthetic community (SynCom) of bacteria (Method 5). Sixteen SynCom strains (table S9) were members of 10 families enriched in endophytic compartments of wildtype plants compared to soil (table S8), and 18 strains matched family OTUs altered in plant defense hormone mutants (tables S6 and S9). Further, 21 of the 38 strains belonged to families that matched endophytic-enriched OTUs from a published census of plants grown in wild Mason Farm soil (*10*).

Both bulk soil and endophytic compartment microbiomes changed over eight weeks following SynCom inoculation (Fig. 2A). Fourteen of the 38 SynCom strains were 'robust colonizers' (fig. S13C; table S9; Method 6h). Six of these 14 are from families predicted to be endophytic-enriched in roots from our Mason Farm soil census (Fig. 2B, overlapping black and orange circles; table S9), corroborating their ability to colonize roots. We identified six 'SynCom EC-enriched' isolates and eight 'SynCom EC-depleted' isolates (Fig. 2C; table S4e; Method 6f). Five of the six 'SynCom EC-enriched' strains belong to families also predicted to be endophytic-enriched in roots from the Mason Farm soil census (Fig. 2B; overlapping orange and red circles; table S9), supporting their categorization as endophytic compartment-enriched families (table S8). Thus, (i) some but not all SynCom isolates robustly colonized the endophytic compartment of host plants in these mesocosms, (ii) the soil and endophytic microbiomes still differed in this context, and (iii) there was considerable overlap in enrichments and depletions between the SynCom and wild soil colonization experimental platforms at the family level.

Seven bacterial isolates were differentially-abundant between wildtype and the defense phytohormone mutants in the SynCom experiments (Fig. 3; Method 6f), including at least one representative from each of the four phyla present in the inoculum (table S9). Six of the seven isolates were either depleted (*Streptomyces* sp. #136, *Chryseobacterium* sp. #8, *Pseudomonas* sp. #50, and *E. coli*) or were sporadic or non-colonizers (*Bacillus* sp. #125 and *Brevundimonas* sp. #374). Four of these six overlapped with families predicted to be differentially-abundant across genotypes in our Mason Farm soil census (Fig. 3B and table S6). And six of seven (all except *Bacillus* sp. #125) were enriched in the defense phytohormone mutants (Fig. 3C). The profiles of differentially-abundant isolates in *pad4* and *sid2* mutants overlapped (Fig. 3C). These data integrate our SynCom experiments with our wild soil census and demonstrate increased abundance in the SA deficient mutants of isolates that were 'sporadic or non-colonizers' across all wild soil endophytic samples. Thus, altering SA production and signaling in the host plant prevents it from fully excluding bacterial taxa that a wildtype plant shuns.

Exogenous SA application to our SynCom experiments also affected bacterial community composition in both bulk soil and endophytic compartment samples (fig. S14A; table S5; CAP 0.3-1.5%; Methods 5b, 6e), consistent with rhizosphere changes in plants treated with SA or JA (*15*, *16*). Two isolates were enriched (*Flavobacterium* sp. #40, (Bacteroidetes) and *Terracoccus* sp. #273, (Actinobacteria)) and one depleted (*Mitsuaria* sp. #370, (β-Proteobacteria)) in the presence of exogenous SA (table S9; fig. S14B,C**;** Method 6f). *Terracoccus* sp. #273 abundance was higher in both SA-treated bulk soil and root endophytic samples (Fig. 4A), and its growth was enhanced by SA in liquid media (Fig. 4B; Method 5c), although its genome contains no obvious SA catabolism genes (taxon IDs in table S9). In contrast, *Mitsuaria* sp. #370 was depleted in endophytic samples treated with SA, and grew less well in its presence (Fig. 4, C and D). *Streptomyces* sp. #303 was weakly enriched in SA-treated samples (Fig. 4E; q-value <0.07), grew on minimal media with 0.5 mM SA as a sole carbon source (Fig. 4F) and contains orthologs to a previously characterized *Streptomyces* SA-degradation operon (fig. S14D; table S9). Thus, the broader effects of SA on microbiome composition consist of both direct and indirect effects on the physiologies of individual community members from limited, specific taxa.

We demonstrate that plant defense phytohormones sculpt the root microbiome in characteristic ways. Elimination of all three defense phytohormone signaling sectors results in abnormal microbial profiles in the root, which may be linked to lowered survival in a wild soil. SA, a key immune regulator in leaves, also modulates the composition of the root microbiome. Plants with altered SA signaling have root microbiomes that differ in the relative abundance of specific bacterial families compared to wildtype. It will be of interest to address whether and how the extra- and intracellular plant immune system receptor systems further condition root bacterial community composition. We demonstrated that different bacterial strains could make use of SA in different ways, whether as a growth signal or as a carbon source. Thus, SA influences the microbial community structure on the root. This may occur by gating bacterial taxa as a consequence of SA function in homeostatic control of immune system outputs, or via as yet undefined effects on microbe-microbe interactions and root physiology. Together, our results show that a central regulator of the plant immune system, largely uncharacterized in the root, directly influences root microbiome composition. Our results could open new avenues for modulating the root microbiome to enhance crop production and sustainability.

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SUPPLEMENTARY MATERIALS

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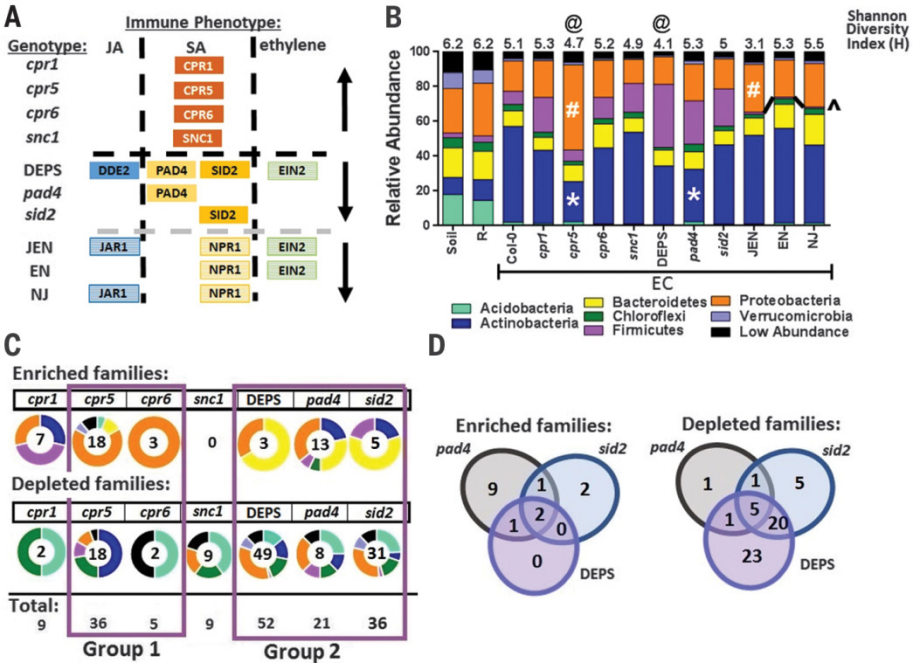

Fig. 1. Defense phytohormone mutants have altered root bacterial communities compared to wild-type plants. (**A**) Jasmonic acid (JA), salicylic acid (SA), and ethylene mutants (names at left) derived from wildtype Col-0. Upward and downward black arrows at right, hyper- and hypo-immune mutants, respectively. (**B**) Phyla distributions were separated into sample fractions (Soil, Col-0 Rhizosphere, R, or Endophytic Compartment, EC) and plant genotypes. Shannon Diversity indices are listed above each bar. * indicates a phylum significantly lower than Col-0 EC at $p<0.001$; # indicates a phylum significantly higher than Col-0 EC at $p<0.05$; ^ indicates that JEN, EN, and NJ Firmicutes relative abundances were significantly lower than Col-0 EC at $p<0.04$; @ indicates Shannon Diversity Index significantly lower than Col-0 EC at $p<0.001$ (all ANOVA with *post hoc* Tukey test). (**C**) The phyla distribution (circles color-coded as in B) of bacterial families identified as either enriched or depleted in ECs of each mutant compared to Col-0. The number of families in each category is noted inside each donut. Groups defined by Monte Carlo testing of Manhattan distances. (**D**) Venn diagram showing the overlap of enriched (left) or depleted (right) Group 2 families from (B).

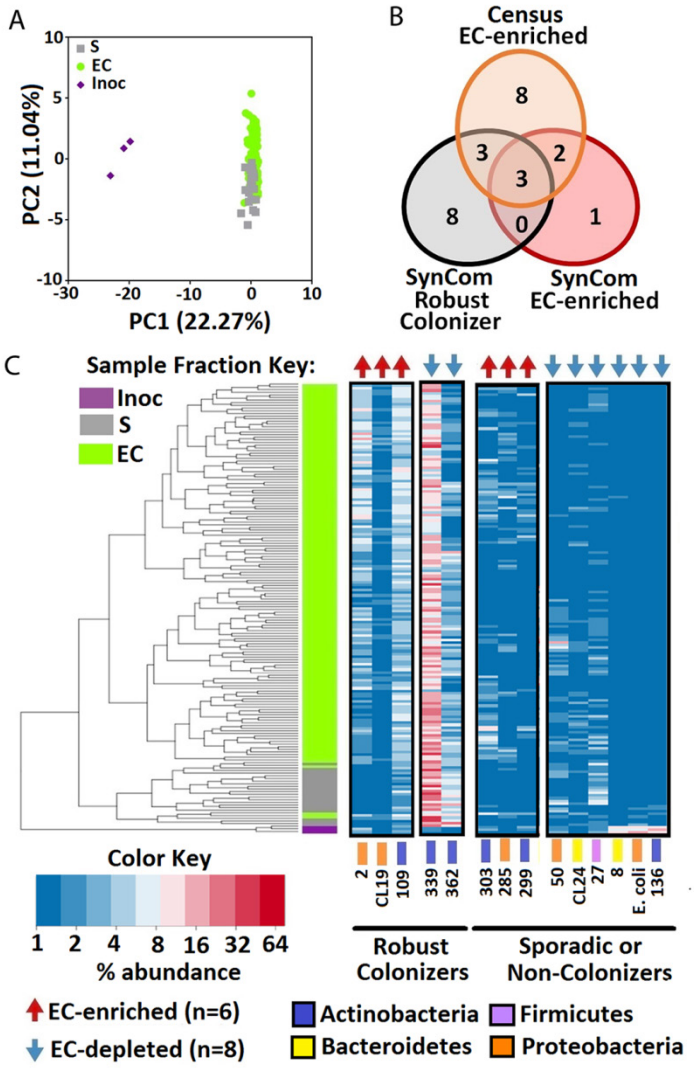

Fig. 2. A 38 member synthetic community recapitulates differentiated microbiome colonization. (**A**) PCA showing the inoculum (purple, diamonds), soil (grey squares) and endophytic compartment (EC;green circles) samples. (**B**) The overlap of synthetic community (SynCom) members that were Robust Colonizers of Col-0 EC (black), EC-enriched (red), or matched EC-enriched families from the census of roots grown in wild Mason Farm soil (orange; from Fig. 1). (**C**) Hierarchical clustering and heat map showing percent abundance (log_2 scale) of selected isolates. Sample clustering split by fraction (left), with EC samples grouping by biological replicate. Isolates are grouped by their presence in the majority of Col-0 EC samples ('Robust colonizers') or absence in the majority of Col-0 EC samples ('Sporadic or non-colonizers'). Isolates color coded to phyla as in Fig. 1. Isolates that were significantly more abundant (red arrows) or less abundant (blue arrows) in EC with respect to bulk soil are denoted along the top.

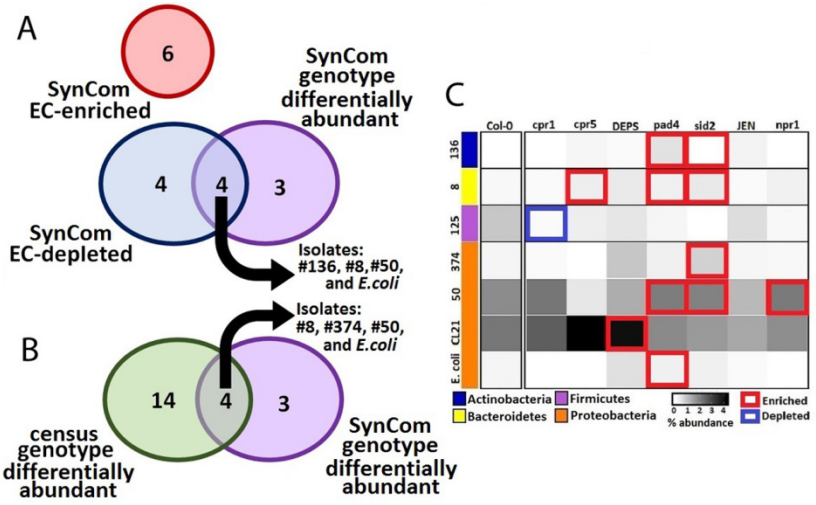

Fig. 3. Defense phytohormone mutants exhibit increased abundance of EC-depleted microbes. (**A**) Overlap of SynCom EC-depleted (from Fig. 2C) and SynCom isolates differentially-abundant in defense phytohormone mutants (SynCom genotype differentially-abundant). No SynCom EC-enriched isolates (from Fig. 2, B and C) were affected by plant genotype. (**B**) Overlap of the same SynCom genotype differentially-abundant isolates from A compared to isolates present in the SynCom from families that were genotype differentially-abundant in the wild soil census (green circle) (from table S8). (**C**) Heatmap of isolates (color-coded by phylum as in Fig. 1) differentially-abundant between defense phytohormone mutants and Col-0. Greyscale shows the mean abundance of the corresponding isolate (rows) in the EC of a given genotype (columns). Genotype differentially-abundant families predicted as enriched or depleted by the ZINB model are boxed in red or blue, respectively (SI, Method 6f).

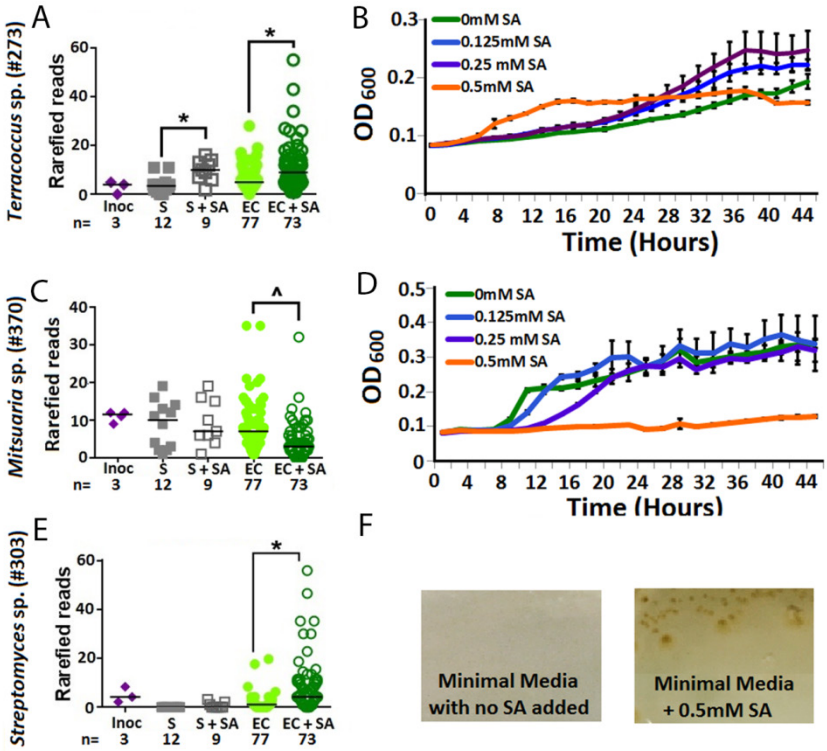

Fig. 4. Salicylic acid directly affects synthetic community isolates. (**A**) Terracoccus sp. (#273) reads from 400 rarefied consensus sequences for: the synthetic community inoculum (purple diamonds), soil (grey squares), and endophytic compartment (EC) samples (green circles) from salicylic acid (SA) treated (open symbols) and untreated (closed symbols) plants. * indicates significantly different between sample treatments at $p<0.006$ by Mann-Whitney test. (**B**) Optical density of Terracoccus sp. (#273) grown in buffered 1/10 LB with 0 (green), 0.125mM (blue), 0.25mM (purple) or 0.5mM (orange) SA added. (**C**) Mitsuaria sp. (#370) reads as in (A). ^ indicates significantly different between EC sample treatments at $p<0.0001$ by Mann-Whitney test. (**D**) Optical density of Mitsuaria sp. (#370) grown as in (B). (**E**) Streptomyces sp. (#303) reads. * indicates significantly different between EC sample treatments at $p<0.001$ by Mann-Whitney test. (**F**) Streptomyces sp. (#303) aggregates in liquid cultures, but grows on minimal media agar with 0.5mM SA as the sole carbon source.