

Endophytes Are Hidden Producers of Maytansine in *Putterlickia* Roots

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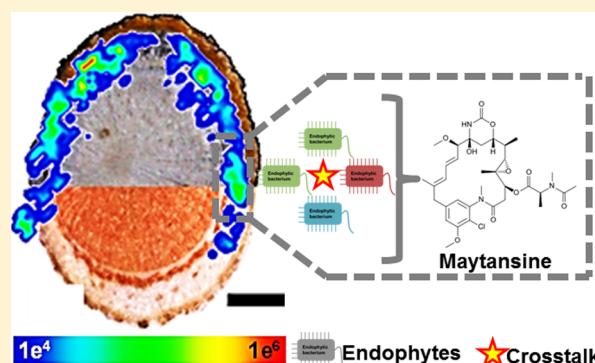
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Supporting Information

ABSTRACT: Several recent studies have lent evidence to the fact that certain so-called plant metabolites are actually biosynthesized by associated microorganisms. In this work, we show that the original source organism(s) responsible for the biosynthesis of the important anticancer and cytotoxic compound maytansine is the endophytic bacterial community harbored specifically within the roots of *Putterlickia verrucosa* and *P. retrospinosa* plants. Evaluation of the root endophytic community by chemical characterization of their fermentation products using HPLC-HRMSⁿ, along with a selective microbiological assay using the maytansine-sensitive type strain *Hamigera avellanea* revealed the endophytic production of maytansine. This was further confirmed by the presence of AHBA synthase genes in the root endophytic communities. Finally, MALDI-imaging-HRMS was used to demonstrate that maytansine produced by the endophytes is typically accumulated mainly in the root cortex of both plants. Our study, thus, reveals that maytansine is actually a biosynthetic product of root-associated endophytic microorganisms. The knowledge gained from this study provides fundamental insights on the biosynthesis of so-called plant metabolites by endophytes residing in distinct ecological niches.



Living in internal plant tissues, endophytic microorganisms maintain associations with their hosts for at least a part of their life.^{1,2} Endophytes are known to function as plant growth and defense promoters by synthesizing phytohormones, producing biosurfactants, averting plant diseases, and aiding in plant tolerance against drought and salinity.^{3,4} Endophytes have been reported to produce a plethora of bioactive compounds, some of which are also present in the host plants such as podophyllotoxin, deoxypodophyllotoxin, camptothecin and structural analogues, and hypericin, to name a few.^{2,4} Furthermore, recent studies have demonstrated that fungi can harbor natural-product-producing bacteria⁵ or that fungi crosstalk with higher plants for the production of important natural products.^{6–8} Yu et al. (2002)⁹ have even hypothesized that certain so-called plant metabolites actually originate from the biosynthetic pathway of endophytes or other plant-associated microorganisms.

Given the central role of chemical crosstalk in plants and endophytes, it is compelling that certain compounds or their precursors formerly believed to be synthesized only by plants or exclusively considered plant metabolites can be produced by endophytes or other plant-associated microorganisms. In one of

our previous studies for example, we showed that the endophytic fungus *Fusarium solani*, isolated from the plant *Camptotheca acuminata*, utilizes endogenous geraniol 10-hydroxylase, secologanin synthase, and tryptophan decarboxylase to biosynthesize precursors of camptothecin. However, to complete the cross-species camptothecin pathway, the endophyte requires host strictosidine synthase.¹⁰ Several other studies have also revealed the existence of microbial producers in various hosts as the source organisms producing compounds encompassing diverse chemical scaffolds. Some prominent examples include the gamma proteobacterial symbiont *Candidatus "Endobugula sertula"* harbored in a marine bryozoan (*Bugula neritina*) that produces bryostatins,¹¹ the *Pseudomonas*-like symbiont of the rove beetles (*Paederus* and *Paederidus*) that produces pederin-type compounds,¹² the cyanobacterial symbiont (*Prochloron didemni*) of the sea squirt *Lissoclinum patella* that produces patellamides A and C,¹³ and the recently reported symbiont (*Entotheonella* spp.) of the

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marine sponge *Theonella swinhonis* capable of producing almost all peptides and polyketides that were isolated earlier from its host.¹⁴

Ansamycins represent a group of natural products having high physiological activity. Ansamycins comprise two structurally discernible groups of compounds, which are characterized by either a benzoic or a naphthalenic chromophore. The respective chromophore is bridged in both cases by an aliphatic polyketide chain.¹⁵ Studies on the biosynthetic pathway of ansamycin antibiotics have established that 3-amino-5-hydroxybenzoic acid (AHBA) is the common specific starter unit of all ansamycins.^{16–21} This molecule, which comprises a biosynthetically unique core structural moiety called mC_nN, containing a six-membered carbocycle (typically aromatic or quinoid) and carrying an extra carbon and a nitrogen in a meta arrangement, serves as the unique starter unit for the biosynthesis of all ansamycins.^{16–21} Maytansinoids are a subgroup of ansamycins belonging to compounds with a benzoic chromophore.²² Maytansinoids were discovered by Kupchan et al.²³ using bioactivity-guided fractionation from extracts of Celastraceous plants such as *Putterlickia verrucosa* or *Maytenus serrata*. This approach led to the discovery of maytansine (Figure 1A), one

of the most potent microtubule-targeting compounds. Presently for example, an antibody–maytansinoid conjugate (Trastuzumab Emtansin, Kadlecyl) is used in therapy to target specifically mamma carcinoma cells.

Forty-eight ansamitocin biosynthetic genes present in *Actinosynnema pretiosum* ssp. *auranticum* and almost all intermediates involved in maytansinoid biosynthesis are known.²² A search for maytansinoid compounds in different organisms revealed that maytansinoids occur not only in Celastraceae but also in Rhamnaceae and Euphorbiaceae,²⁴ certain mosses,^{25,26} and even in bacteria (*A. pretiosum*).²⁷ The disjoint occurrence of maytansinoids is intriguing from the chemotaxonomic point: Why is maytansine present in unrelated organisms? Indeed an infective microorganism could be responsible for the presence of maytansine in plants.^{28,29} This would be in agreement with the fact that an intensive search for the AHBA synthase gene (*asm24, rifK*) in sterile plant cell suspension cultures of *P. verrucosa* failed to give any indication that biosynthesis of maytansine is accomplished by plants.^{28,29} Furthermore, analysis of the DNA isolated from microorganisms of the rhizosphere after physical breakup of cell walls in a freeze–thaw procedure³⁰ provided DNA with sequences matching the variable V₃, V₄, and V_{6–9} sites of 16S rDNA as well as the 16S–23S internal transcribed spacer (ITS) of *A. pretiosum*.²⁹ It is for this reason that an infective microorganism(s) responsible for the presence of maytansine in plants has been postulated,^{28,29} which could even be an endophyte or endophytic community. Moreover, 16S rRNA gene sequences were found in the rhizosphere of maytansine-containing plants matching the 16S rRNA sequences of the ansamitocin-producing bacterium *A. pretiosum*.²⁹ This indicated that root-associated bacteria of *P. verrucosa* might be responsible for production of maytansine.

In this study, we show that maytansine is biosynthesized by the endophytic bacterial community harbored specifically within the roots of *Putterlickia* plants. Using the combination of high-performance liquid chromatography high-resolution mass spectrometry with electrospray ionization (HPLC-ESI-HRMS²) and matrix-assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HRMS), it was revealed in high spatial resolution that maytansine is located and typically accumulated in the root cortex of both *Putterlickia verrucosa* and *P. retrospinosa* plants, but not in other parts of the roots or in the shoots (where it is variable and dispersed). Evaluation of the endophytic bacterial community of the roots by chemical characterization of their fermentation products in combination with a selective microbiological assay using the maytansine-sensitive type strain *Hamigera avellanea* (syn. *Penicillium avellaneum*) revealed independent biosynthesis of maytansine by the bacterial community (*in vitro*, outside the host plant). This was further confirmed by the presence of AHBA synthase genes in the root endophytic communities. This work, thus, demonstrated the localization of endophytes responsible for the biosynthesis of maytansine specifically in the roots and further accumulation pattern of the produced maytansine especially in the root cortex.

Our preliminary investigation of the shoots and roots of maytansine-containing plants revealed that the bulk of maytansine was found to explicitly accumulate within the roots independent of the external environmental conditions (such as sampling time, soil, climatic conditions, etc.). This was observed for both *P. verrucosa* and *P. retrospinosa*. Only minor

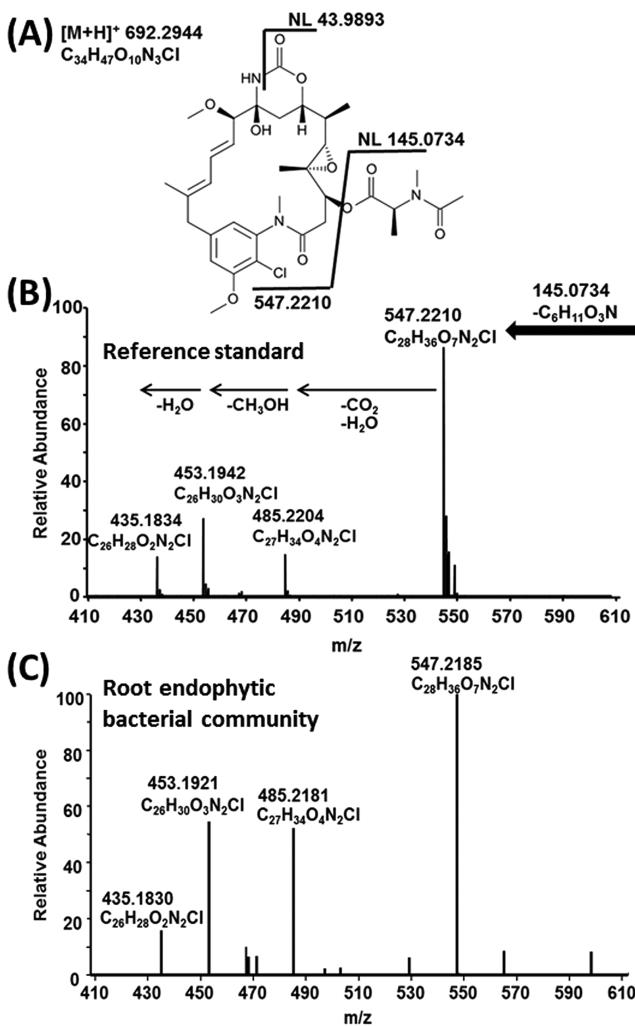


Figure 1. Structural formula and HRMS² fragmentation of maytansine. (A) Structure of maytansine. (B) HRMS² fragmentation of authentic maytansine standard. (C) Representative HRMS² fragmentation of maytansine produced by root endophytic bacterial community.

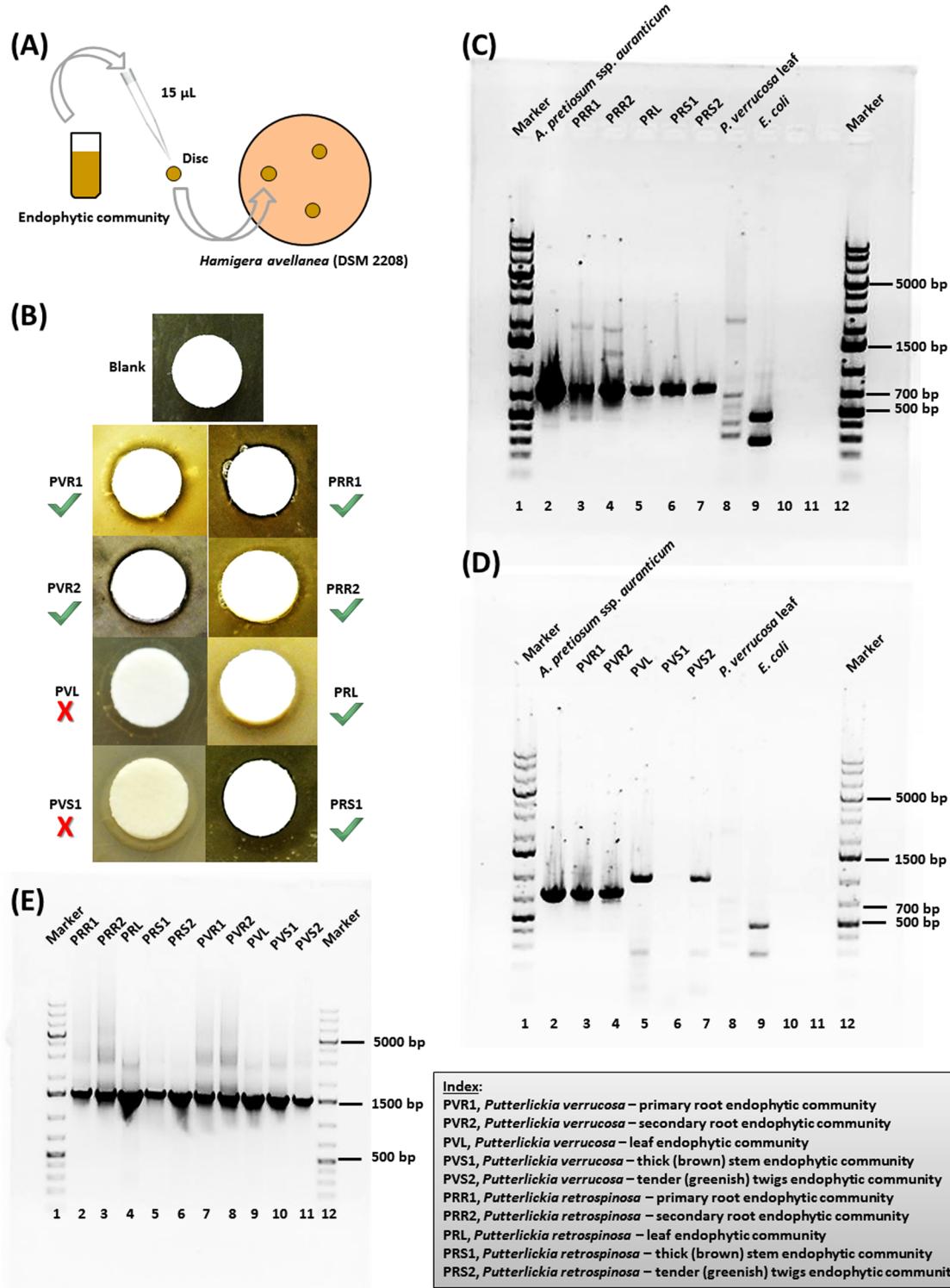


Figure 2. Microbiological assays and molecular analyses of the isolated endophytic communities. (A) Schematic representation of the plate-based microbiological assay to detect maytansine production by the endophytic microbial community by challenging them with the maytansine-sensitive type strain *Hamigera avellanea* (syn. *Penicillium avellaneum*; DSM 2208). (B) Results of the endophyte community-*H. avellanea* assay. Observed zones of inhibition (ZOIs) are additionally shown with a green tick-mark and the ones without ZOI by a red cross-mark. (C) Stained agarose gels of purified PCR-amplified products of AHBA synthase genes (spanning around 755 bp) in *Putterlickia retrospinosa* bacterial endophytic community. *Actinosynnema pretiosum* ssp. *auranticum* (DSM 44131) is the positive control; *Putterlickia verrucosa* leaf and *Escherichia coli* (DSM 682) are negative controls. (D) Stained agarose gels of purified PCR-amplified products of AHBA synthase genes (spanning around 755 bp) in *P. verrucosa* bacterial endophytic community. Positive and negative controls are the same as in panel C. (E) Stained agarose gels of purified PCR products of 16S rRNA analyses of bacterial endophytic communities of *P. retrospinosa* and *P. verrucosa* (spanning around 1500 bp).

but variable amounts were detectable in the shoots. It is well known that production, accumulation, and transport of

phytochemicals within plants are influenced by biotic and/or abiotic environmental conditions irrespective of their bio-

synthetic pathways. Our earlier work corroborated this for polyketides,³¹ lignans,³² and even alkaloids.³³ Therefore, in light of our preliminary data and earlier evidence that sterile *Putterlickia* plant cell cultures are devoid of endophytes and could not produce maytansine,^{28,29} we hypothesized that an endophytic microbial source specific to the roots is likely to be responsible for the biosynthesis of maytansine. To further test this possibility, freshly collected pieces of primary and secondary roots of both *P. verrucosa* (coded PVR1 for the primary root and PVR2 for secondary roots) and *P. retrospinosa* (coded PRR1 for the primary root and PRR2 for secondary roots) plants were freed from the rhizosphere microbiota by surface sterilization in a process that saved the endorhizal microbial community. These pieces of roots were then thoroughly macerated (primary and secondary roots separately) and seeded into optimized *Streptomyces* broth medium (SM) suitable for triggering natural product formation in *Streptomyces* species. The endophytic community of the stems (for *P. verrucosa*, coded PVS1 for the thick, main stem and PVS2 for the thin, tender twigs including the thorns; for *P. retrospinosa*, coded PRS1 for the thick main stem and PRS2 for the thin, tender twigs including the thorns) and leaves (coded PVL and PRL for leaves of *P. verrucosa* and *P. retrospinosa*, respectively) of both plants were isolated in parallel. The cultures were extracted and analyzed by HPLC coupled to a high-resolution mass spectrometer. The resulting measurements of extracts (primary as well as secondary root bacterial endophytic community) of both plants exhibited the characteristic isotopic pattern of a chloric compound with a signal at *m/z* 692.29412, which was in very good agreement with the calculated mass of the quasi molecular ion $[C_{34}H_{47}O_{10}N_3Cl]^+$ of maytansine. Comparison of the chromatographic and spectroscopic data with an authentic standard confirmed production of maytansine by the bacterial endophytic community of both the primary and secondary roots of both plants (Figure 1). Furthermore, the fragmentation (HRMS²) of the protonated maytansine revealed the characteristic loss of the ester side chain C₆H₁₁O₃N to a fragment with *m/z* 547.2210, which could be assigned to the macrolide moiety (Figure 1B,C). However, the endophytic bacterial community isolated from the stems and leaves could not produce maytansine. Furthermore, production of maytansine was not observed when the roots or shoots were completely sterilized prior to inoculation (negative control). We concluded that the primary and secondary roots of both experimental plants contain a tissue-specific endophytic bacterial community that produces maytansine. Moreover, in order to confirm the host-specificity and systemic nature of the root endophytic bacterial community, we bioprospected the same plants twice (in 2013 and 2014) and independently repeated the complete experimental setups (from isolation of endophytes to confirmation of production of maytansine by the root-specific endophytes by LC-HRMS²). For both plant sampling time points, it was revealed that maytansine could be biosynthesized by the endophytic bacterial community harbored specifically within the roots of *P. verrucosa* and *P. retrospinosa* plants.

We further examined how maytansine is produced by the root endophytic microbial community. We combined two approaches in parallel. First, we used a well-established, simple, yet effective plate-based microbiological assay to detect maytansine production³⁴ by challenging the endophytic microbial community with the maytansine-sensitive type strain *Hamigera avellanea* (syn. *Penicillium avellaneum*; DSM 2208)

(Figure 2A). Second, using the knowledge that the common starter for all ansamycins, AHBA (comprising the unique mC₇N structural moiety), is biosynthesized by the highly specific and essential enzyme AHBA synthase,^{16–21} we evaluated the endophytic communities for the presence of genes encoding this enzyme. Specifically, we selected a primer pair based on the two conserved amino acid regions (FEREFA and HYMAM) of five AHBA synthase genes, namely, *asnF*, *asm24*, *mitA*, *napF*, and *rifK*, that can amplify the conserved 755 bp fragment of AHBA synthase genes coding for their enzymatic activity.¹⁸ As expected, the degenerate primers amplified the AHBA synthase genes in the positive control *A. pretiosum* subsp. *auranticum* (DSM 44131) with optimized PCR conditions (Figure 2C,D). The (ca.) 755 bp product, thus obtained, was sequenced. The respective protein sequences corresponding to the amplified product were identified and matched using the UniProtKB, showing 100% homology to AHBA synthase (EMBL AAC13997.1 and UniProt identifier Q44131). Two negative controls were used: the leaf of *P. verrucosa* served as a plant control, and *Escherichia coli* (DSM 682) served as a bacterial control. As expected, none of them produced the desired amplification product (Figure 2C,D). Thereafter, each endophytic community template DNA was subjected to PCR amplification using optimized PCR conditions with the same degenerate primer pair. The root endophytic communities of both plants, namely, PVR1, PVR2, PRR1, and PRR2, produced amplified products of expected sizes (Figure 2C,D). Concomitantly, a clear zone of inhibition (ZOI) could be observed when these communities inhibited the growth of *H. avellanea* type strain in the plate assay (Figure 2B). As expected from the fermentation results (LC-HRMS) and microbiological assay (Figure 2B), the leaf and stem communities of *P. verrucosa* did not amplify the desired sequence (Figure 2C,D and Table S1, Supporting Information). Interestingly, however, the endophytic community harboring the leaves and stems of *P. retrospinosa* also showed an amplified product similar to the root communities (Figure 2C,D), even though they did not show production of maytansine under standard *in vitro* fermentation conditions (i.e., <LOD). The microbiological assay also revealed ZOIs for these communities (Figure 2B). The positive sequences were translated and matched against the UniProtKB database to identify the maximum homology with the respective coding protein sequences. PRR1 showed 100% homology with AHBA synthase (EMBL AAC13997.1 and UniProt identifier Q44131). PRR2, PRL, PRS2, and PVR2 indicated 99% homology, whereas PVR1 and PRS1 exhibited 98% homology with AHBA synthase (EMBL AAC13997.1 and UniProt identifier Q44131) (Table S1, Supporting Information). The sequences of the amplified PCR products have been submitted at the EMBL-Bank. Thus, far, we could conclude that the root endophytic communities of both the plants contain a molecular blueprint for the production of maytansine. These results, combined with the chemical characterization of their fermentation products (Figure 1), demonstrated that the root endophytic communities of both plants can indeed biosynthesize maytansine. Finally, our study showed that *P. verrucosa* leaves do not contain AHBA synthase genes, corroborating the earlier study on its sterile plant cell suspension cultures,^{28,29} thereby ratifying that maytansine could not be a “carryover” from the host plant into the root endophytic community biomass. It was interesting to note that the AHBA synthase genes remain “cryptic” in the leaf and stem endophytic communities of *P. retrospinosa*, which

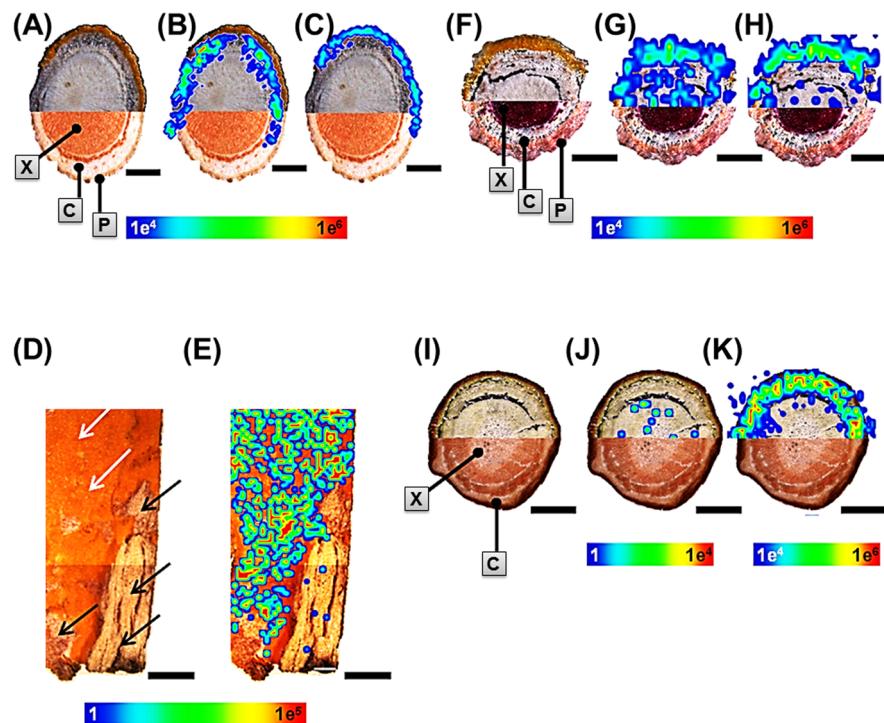


Figure 3. Localization of maytansine by MALDI-imaging-HRMS in *Putterlickia verrucosa* and *P. retrospinosa* plants. (A) Transverse section (TS) of *P. verrucosa* primary root. Lower half of the cross-section stained with phloroglucinol/HCl in order to visualize anatomical structures (X, xylem; C, cortex; P, periderm). (B) Localization of maytansine in the cortex ($[C_{34}H_{46}O_{10}N_3Cl+K]^+$; $m/z = 730.250 \pm 0.001$). (C) Localization of phosphatidylcholine in periderm ($[C_{42}H_{80}O_8NP+Na]^+$; $m/z = 780.552 \pm 0.001$). (D) Longitudinal section (LS) of *P. verrucosa* primary root. White arrowheads show tissue layers exposed within the cortex, and black arrowheads show the inner xylem tissues. (E) Localization of maytansine only in the cortex and not in the periderm or xylem tissues ($[C_{34}H_{46}O_{10}N_3Cl+K]^+$; $m/z = 730.250 \pm 0.001$). (F) TS of *P. retrospinosa* primary root. Lower half of the cross-section stained with phloroglucinol/HCl in order to visualize anatomical structures (X, xylem; C, cortex; P, periderm). (G) Localization of maytansine mainly in the cortex with minor amount in the xylem ($[C_{34}H_{46}O_{10}N_3Cl+K]^+$; $m/z = 730.250 \pm 0.001$). (H) Localization of phosphatidylcholine in the cortex ($[C_{42}H_{80}O_8NP+Na]^+$; $m/z = 780.552 \pm 0.001$). (I) TS of *P. retrospinosa* stem. Lower half of the cross-section stained with phloroglucinol/HCl in order to visualize anatomical structures (X, xylem; C, cortex). (J) Localization of only a trace amount of maytansine in the xylem ($[C_{34}H_{46}O_{10}N_3Cl+K]^+$; $m/z = 730.250 \pm 0.001$). (K) Localization of phosphatidylcholine in the cortex ($[C_{42}H_{80}O_8NP+Na]^+$; $m/z = 780.552 \pm 0.001$). Scale bars for panels A–C and F–K represent 500 μ m, and those for panels D and E represent 2000 μ m. The intensity scales, which were different for each setup, are represented as color-coded relative intensities below the respective panels.

can be activated by certain biotic and/or abiotic selective pressures (as exemplified when they were challenged with *H. avellanea*; see Figure 2B).

We then aimed to elucidate the phylogenetic relationship between the different endophytic communities isolated from the leaves, stems, and roots of both plants. The 16S rRNA amplification of the endophytic communities of *P. retrospinosa* and *P. verrucosa* resulted in desired PCR products of approximately 1500 bp size (Figure 2E). The products were further sequenced from both directions and aligned using EMBOSST-Needle pairwise sequence alignment. The multiple sequence alignment of all the bacterial endophytic communities (PRR1, PRR2, PRL, PRS1, PRS2, PVR1, PVR2, PVL, PVS1, and PVS2) using T-Coffee, combined into one final alignment, showed the phylogenetic correlation between the communities based on their 16S rRNA regions (Figure S1, Supporting Information). The final output of the alignment indicated the consistency of the endophytic sequences ranging from the most strongly supported sequence(s) compared with the T-Coffee library (highlighted in dark red) to the least supported one(s) (highlighted in a gradient from light red to blue). The total consistency value was found to be 890, whereas the individual consistency scores were as high as 92 (PVS2) and 90 (PVR2) followed by almost all the others, with a score of 88. The only exception was PRR2, with a low consistency score of 39. A high

score represented total agreement with the respective sequence and its associated library. Thus, higher scores indicated higher accuracy of alignment. In this work, alignment of all the endophytic community with the only exception being PRR2 resulted in high consistency scores, thereby highlighting higher accuracy with the T-Coffee primary library and its relevance in multiple sequence alignment. Further, these aligned outputs were converted into a phylogenetic tree, taking branch lengths into consideration. Using Drawgram, a phenogram-like rooted phylogenetic tree was constructed using centered ancestral nodes (Figure S2, Supporting Information). The phylogenetic tree represented the correlation between the communities on horizontal levels. The phenogram clearly indicated the different nodes of connection between the communities. The communities of primary and secondary roots of *P. verrucosa* were closely related. However, for *P. retrospinosa*, although the internodes were connected, the root communities were quite distant. This highlighted the fact that there could be a similarity between PRR1 and PRR2 on a higher level of taxonomic classification. The phenogram also pointed towards the probable similarity between PRS2 and PVL communities, and PRS1 and PVS2 communities, respectively. PRL was connected to PRS1 and PVS2 communities on an internodal level. Interestingly, the PVS1 community was completely distinct and separate from all the other communities, taking the central

ancestral node into consideration. Further, the distance of PVS1 from the central ancestral node was the same as that of PRL on the other side of the centered node. The multiple alignment sequences with consistency scores and phylogenetic tree represented the species-level relatedness between the different endophytic bacterial communities harbored in different tissues of *P. verrucosa* and *P. retrospinosa* plants.

Since the biosynthesis of maytansine was performed only by root-specific endophytic bacteria, we aimed to pinpoint the tissue-specific site of accumulation of maytansine in order to understand its possible ecological role(s). We investigated the transverse and longitudinal sections of *Putterlickia* plants using MALDI-imaging high-resolution mass spectrometry. The resulting signal intensities were made visible by a color coding system (Figure 3) ranging from blue (low intensity) to red (high intensity). Mass detection was not only confined to maytansine ($[C_{34}H_{46}O_{10}N_3Cl+K]^+$, m/z 730.250) but also included a phosphatidylcholine (1,2-diacyl-sn-glycero-3-phosphocholine-34:2, $[C_{42}H_{80}O_8NP + Na]^+$, m/z 780.552), which was used as a control for the specificity of the analytical technique. The roots and stems were stained with phloroglucinol/HCl in order to visualize anatomical structures within the roots (Figure 3 also shows the stained lower halves of the cross sections). Figure 3A shows a cross section of *P. verrucosa* primary root and the same sample after detection of maytansine (Figure 3B) and phosphatidylcholine (Figure 3C). Using imaging mass spectrometry, we clearly distinguished in high spatial resolution between maytansine located in the cortex and phosphatidylcholine in the periderm. A longitudinal section of a *P. verrucosa* root after exposure to the MALDI-imaging-HRMS system (Figure 3E) showed maytansine-containing tissue layers exposed within the cortex and viewed from the center of the root (Figure 3E). Removal of the cortex from the intact root resulted in a sample in which also tissue from deeper layers (xylem) present within the root was removed (black arrowheads in Figure 3D). Deeper layers did not contain maytansine (compare Figure 3D and E). Again, maytansine occurred in the cortex (white arrowheads) but not in deeper layers such as the xylem (black arrowheads). The *P. retrospinosa* root (Figure 3F–H) and shoots (Figure 3I–K) were subsequently investigated. The phosphatidylcholine derivative could be detected mainly in the cortex of both root (Figure 3H) and shoot (Figure 3K), but maytansine was primarily localized in the root cortex (Figure 3G). Unlike in *P. verrucosa*, traces of maytansine were also seen in the xylem of the root (color intensity blue) (Figure 3G) and shoot (Figure 3J). From the LC-HRMS results of the fermentation products, we initially predicted that some maytansine from the root cortex might be translocated with the plant transpiration stream into the xylem of the above ground twigs, leading to the presence of very low and sparse amounts of maytansine in the shoot xylem (Figure 3J). However, the presence of AHBA synthase genes in the leaf and stem endophytic communities of *P. retrospinosa* hinted toward the possibility of sporadic production of maytansine by the endophytes, only when suitably triggered (possibly as chemical defense).

The rhizosphere is a microbial “hot spot” with multifaceted microbe–microbe and microbe–plant interactions. The *in planta* environment also forms a nutrient-rich niche for microorganisms, as evident from the release of exudates from the root system. It is generally accepted that natural products play an important role in this setting.^{29,35–37} The biosynthesis of these natural products might have gradually evolved over

time to benefit the “source” organisms and help them to maintain or improve their fitness in the environment.^{38,39} It is highly plausible that maytansine may also play a significant role in the response of *Putterlickia* plants when challenged by pathogens, as indicated by the presence of AHBA synthase genes in the leaf and stem endophytic communities of *P. retrospinosa*. While arresting cell division, maytansine binds to the β -subunit of tubulin²² and is effective against eukaryotic systems such as protozoa,²⁸ yeasts, fungi, insects, and plants.⁴⁰ The accumulation of maytansine in the cortex of the root (Figure 3) is possibly the preferred site that can prevent pathogen intrusions through plant roots.

The confirmation of production of maytansine by endophytic microbes associated with *Putterlickia* plants opens up further avenues for investigating whether maytansine is produced by a single endophyte or by crosstalk between two or more endophytes in a community, and if it might be possible to separate the maytansine producer(s) from the community without hindering their biosynthetic capabilities. Given the antimicrobial and antineoplastic action of maytansine, it is conceivable on one hand that a single organism producing maytansine could essentially disrupt the entire community and concomitantly the balance between endophytes and among the endophytes and host plant(s). On the other hand, a more feasible scenario could be a multifaceted crosstalk (suitable chemical triggers, production of precursors, and so on) between two or more organisms within the root endophytic microbial community in that particular ecological niche to produce maytansine. Furthermore, it would be interesting to note the genus- and species-level similarities between the reported ansamycins and the maytansine-producing endophyte(s), if any. Our work reported here provides a scientific handle for further investigation along the above lines, with the broader goals of understanding the role of plant colonization by the maytansine-producing microbes, overall flux operating between the plant and maytansine-producing microbial interface, and the overall ecological role of maytansine production by plant-associated microbes (given its antimicrobial and antineoplastic action). Further studies along these lines are under way.

Identification and Quantitative Determination of Maytansine by HPLC-ESI-HRMS⁷. The origin, authentication, and maintenance of the plants have been described by Pullen et al. (2003).²⁸ The air-dried plant material (1 g) was cut and extracted with MeOH (3 mL) at room temperature twice for 24 and 48 h, respectively. The combined extracts were analyzed by means of HRMS as described earlier.²⁹

MALDI-Imaging-HRMS to Visualize Accumulation of Maytansine in Plant Roots and Shoots. For MALDI imaging, 10 mm primary root or main stem material was excised from the fresh plants using sterile tweezers and scissors. Excised samples were stored at -20°C until used in the experiment. The tissue was cut to $40\ \mu\text{m}$ sections in a Thermo Scientific HM550 cryostat at -20°C box temperature and -10°C sample temperature. MALDI-imaging-HRMS was performed as described before.^{41,42} The sections were attached to sticky, double-sided tape (3M), thawed, and dried in a vacuum for 30 min. Thereafter 2,5-dihydroxybenzoic acid (Sigma-Aldrich; 30 mg mL^{-1} , 50% MeOH, 0.2% TFA) was applied using a Bruker Daltonics ImagePrep device. Samples were measured with a Thermo Scientific MALDI-LTQ-Orbitrap mass spectrometer with $50\ \mu\text{m}$ lateral resolution and a nominal spectral resolution of 60 000 at m/z 400. The scan area ranged from m/z 600 to 800 to include lipid signals

for comparison. Per scan, 1×10^6 ions were collected (automatic gain control target) by adjusting the number of shots per position at a constant laser power of $30 \mu\text{J}$. Spectra were internally recalibrated (lock mass) using the exact mass of DHB- $2\text{H}_2\text{O}$ at m/z 273.03936. The data were analyzed and images were created using Thermo Image Quest software (v.1.0.1).

For identification of anatomical features, embedded sections were cut in fully optimal cutting temperature freezing medium (OCT), and the OCT was washed off with distilled water. Sections were incubated in 4% phloroglucinol (1,3,5-trihydroxybenzene; Sigma-Aldrich) in ethanol for 10 min and developed with 25% HCl for 2 min. Afterward, the stained sections were dried and attached to glass slides using double-sided tape. The photographs and the MALDI image of a respective part were used for the overlay images.

ASSOCIATED CONTENT

Supporting Information

General experimental procedures; the multiple sequence alignment of all the bacterial endophytic communities; phylogenetic tree representing the correlation between the tested/evaluated endophytic communities; overview AHBA synthase genes found in the different endophytic communities isolated from different tissues of *Putterlickia* plants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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