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A study of the bacterial community in the root system of the maytansine containing plant *Putterlickia verrucosa*

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ABSTRACT

Maytansinoid compounds are ansa antibiotics occurring in the bacterium *Actinosynnema pretiosum*, in mosses and in higher plants such as *Putterlickia verrucosa* (E. Meyer ex Sonder) Szyszyl. The disjunct occurrence of maytansinoids has led to the consideration that plant-associated bacteria may be responsible for the presence of maytansinoids in *P. verrucosa* plants. Investigation of the bacterial community of this plant by molecular methods led to the observation that *A. pretiosum*, a maytansine-producing bacterium, is likely to be an inhabitant of the rhizosphere and the endorhiza of *P. verrucosa*.

1. Introduction

Ansamycins are a group of bacterial natural products characterized by strong physiological activities, an unusual structure and a biosynthetic pathway comprising a novel aromatisation process, the amino shikimate pathway (Kibby et al., 1980; Floss, 2006). Often ansamycins have an aliphatic ansa chain linked to either a benzenic or naphthalenic chromophore. Maytansine (**1**) (Fig. 1), ansatrienine, mitomycin and geldanamycin belong to the former, rifamycin, streptovaricin, naphthomycin and rubradirin belong to the latter group of ansamycins (Gräfe, 1992; Higashide et al., 1977; Prelog and Oppolzer, 1973; Wehrli, 1977). Naphthalenic ansamycins exhibit antibiotic activities against gram positive bacteria, especially *Mycobacteria*, rifampicin being a semisynthetic naphthalenoid ansamycin employed against tuberculosis and leprosy. Benzenoid ansamycins often exhibit antineoplastic activity. Among these are the ansamitocins which are called maytansines when they are present in higher plants (Cassady et al., 2004; Higashide et al., 1977; Komoda and Kishi, 1980; Kupchan et al., 1974; Smith and Powell, 1984; Reider and Roland, 1984). Maytansines were first isolated from higher plants by (Kupchan and associates, 1972a,b, 1977) and later also found to be produced by the bacterium *Actinosynnema pretiosum* isolated from a *Carex* species (Higashide et al., 1977). Two sub-species of *A. pretiosum* were described, *A. pretiosum* ssp. *pretiosum* and *A. pretiosum* ssp. *auranticum*. Both sub-species produce ansamitocins e.g. ansamitocin P-3 (Hasegawa et al., 1983; Tanida et al., 1981). Maytansines were isolated from *Maytenus ovatus* (Kupchan et al., 1972a), *Maytenus buchananii* (Kupchan et al., 1972b, 1974), and *Putterlickia verrucosa* (E. Meyer ex Sonder) Szyszyl. (Kupchan et al., 1977), plants belonging to the family *Celastraceae*. Structurally maytansines and ansamitocins differ mainly by the ester side chain at position 3 (Fig. 1). Maytansinol and ansamitocin P-0 as well as maytanacin and ansamitocin P-1, however, represent identical structures.

The biosynthesis of ansamycins includes a novel aromatisation process leading to 3-amino-5-hydroxybenzoic acid (AHBA). Enzymatic conversion of 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP) to AHBA was the first elucidated reaction sequence in an aromatisation process which involves 5-deoxy-5-amino-3-dehydroquinic (aminoDHQ) and 5-deoxy-5-amino-3-dehydroshikimic acid (aminoDHS) (Kim et al., 1992, 1996). The latter compound is converted in a pyridoxal phosphate dependent reaction via a α,β-dehydration and stereospecific 1,4-enolisation to AHBA (Kim et al., 1998). The enzyme catalyzing this reaction was purified to homogeneity and crystallized. While X-ray crystallographic data clarified mechanistic aspects of this enzyme (Eads et al., 1999) a reverse genetics experiment gave access to the encoded gene (Kim et al., 1998; Yu et al., 2001). Chromosome walking and gene inactivation led to the discovery of a type I modular polyketide synthase which uses the activated AHBA as a starter molecule to build the ansa chain from three propionate, three acetate units and methoxymalonate giving rise to the basic ansamitocin skeleton (August et al., 1998; Kibby et al., 1980). One step

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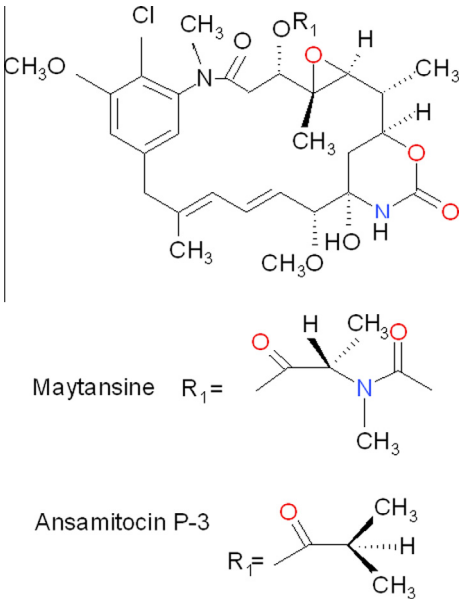

Fig. 1. Structural formulae of a plant ansamitocin (maytansine) and a bacterial ansamitocin (ansamitocin P-3).

in these extensions supplies the unusual "glycolate" unit from an activated methoxymalonate (Caroll et al., 2002; Kato et al., 2002; Wenzel et al., 2006). These data did not yet give an explanation for the origin of the early steps responsible for the introduction of the nitrogen into this pathway but kanosamine 6-phosphate (Arakawa et al., 2002; Guo and Frost, 2002a, 2002b) proved to be the pivotal starting material. Proansamitocin is the first ansamitocin type metabolite in the biosynthetic pathway (Cassady et al., 2004). The sequence by which this intermediate is decorated by substitution steps has been elucidated by gene inactivation and feeding experiments (Moss et al., 2002; Cassady et al., 2004).

One of the unusual features of the genes encoding the ansamitocins is that they are organized in two subclusters separated by 30 kilobases apparently nonfunctional DNA. A total of 48 genes are responsible for ansamitocin biosynthesis (Yu et al., 2002). A summary of the biology, chemistry, metabolism, and biosynthesis of ansamitocin has been published (Cassady et al., 2004).

Another unusual feature of the ansamitocins is that they occur in bacteria, higher plants belonging to the families *Celastraceae*, *Rhamnaceae* and *Euphorbiaceae* (Cassady et al., 2004) and even in mosses, namely *Claopodium crispifolium* and *Anomodon attenuatus* (Thuidiaceae) (Suwanborirux et al., 1990), *Isothecium subdiversiforme* (Lembophyllaceae) and *Thamnobrium sandei* (*Neckeraceae*) (Sakai et al., 1988). The disjunct occurrence of maytansinoids (ansamitocins) in higher plants, mosses and bacteria seems to contradict the principle of chemotaxonomy that identical or at least similar natural products have a common evolutionary history and thus occur in taxonomically related organisms. The fact that maytansinoids occur in some but not all individual *P. verrucosa* plants may indicate, however, that bacterial or fungal endophytes or epibionts are responsible for the presence of maytansinoids in higher plants (Pullen et al., 2003). The present article contributes to the solution of this problem.

2. Results

2.1. Nondetectability of an AHBA synthase gene in cultured P. verrucosa cells

A plant cell suspension culture (Pullen et al., 2003) was chosen in order to test the possibility that cells of the plant *P. verrucosa* would be capable to synthesize maytansine. A cell culture seemed to be a particularly suitable source of DNA because they are usually axenic and a contamination by a plant associated microorganism would be avoided. After extraction of DNA PCR reactions were carried out with oligomers targeted to codons of amino acid residues known from X-ray crystallographic data (Eads et al., 1999) to have an essential function in the catalytic process of the AHBA synthase. Codons of the following essential amino acid residues were included: Asp 159, Gly 62, Thr 63, Leu 66, Phe 88, Ala161, His 162, Ser 183, Arg 236. The derived codons were adjusted in position 3 to the codon usage of the *Putterlickia* plant which was determined by sequencing of the two 3-deoxy-*arabino*-heptulosonate 7-phosphate synthase genes (E.C.4.1.2.15), initiating the shikimic acid pathway. Six forward and six reverse oligonucleotides targeted to the AHBA synthase gene were employed in 22 combinations. The DNA fragments generated in this way had a length of 99–531 base pairs (exons only). As primer also the bacterial oligomers also the bacterial oligomers AHBA1f and AHBA2r (Pullen et al., 2003) were employed. After the PCR reactions, DNA fragments were isolated and sequenced. In every case, no sequence was found that showed any homology to the bacterial AHBA synthase gene in maytansine biosynthesis (Cassady et al., 2004; Yu et al., 2002). This result matched our previous observations in which oligomers based on conserved regions of the bacterial AHBA synthase gene (asm 24) and a late enzyme in maytansine biosynthesis, amide synthase (asm 9) was employed (Pullen et al., 2003).

The chromosomal DNA extracted from cultured *Putterlickia* cells was also subjected to Southern blot experiments (Pullen et al., 2003). After enzymic hydrolysis, the plant DNA was probed with DNA sequences representing the same bacterial genes as above (asm9 and asm24). No hybridisation was observed. Thus, no evidence for biosynthesis of maytansine by plant cells of *P. verrucosa* was found.

2.2. Search for a rhizosphere associated microorganism capable to synthesize maytansine

The disjunct occurrence of maytansinoids could be explained by the assumption that the *P. verrucosa* and taxonomically related *Maytenus* plants are colonized by *A. pretiosum*, the bacterial producer of maytansine which had been isolated from an undetermined *Carex* species (Higashide et al., 1977). The maytansine producing bacterium was assumed to be root-associated (Pullen et al., 2003; Cassady et al., 2004). *P. verrucosa* was chosen as the main experimental object because among different maytansine containing plant species, the ansamitocins are most abundant in this plant. Initially, isolation of the maytansine producing bacterium from the root system was attempted on a medium supporting growth of actinomycetes. Single cell colonies were checked for the presence of the AHBA synthase gene by PCR (Pullen et al., 2003). In this way, two *Streptomyces* bacteria (tentatively annotated R12 and R27a) were found which hitherto were not known to contain an AHBA synthase gene. Both strains, however, did not produce detectable amounts of maytansine. A third bacterium was also found which was newly described and named *Kitasatospora putterlickiae* (Groth et al., 2003). In spite of the presence of a AHBA synthase gene, this bacterial species did not seem to produce maytansine (**1**).

We had to take into account that a maytansine producing strain would be nonculturable as are many microorganisms present in their natural habitat (Amann et al., 1995). We therefore set out to search for the maytansine producer by molecular methods. This approach avoids cultivation of the sought-after organism. We compared the 16S rDNA genes of the maytansine producing bacteria *A. pretiosum* ssp. *pretiosum* (DSM 44132) and *A. pretiosum* ssp. *aurantiacum* (ATCC 31565) and a close relative of these strains, *Actinosyn-*

nema mirum (DSM 43827) with rDNA genes found in the rhizosphere of *P. verrucosa*. To this end, highly conserved and variable regions of the rDNA genes (Neefs et al., 1993; Schmalenberger et al., 2001) were amplified by different methods (Table 1) and the resulting PCR products analyzed by single strand conformation polymorphism (SSCP) followed by DNA sequencing. These methods are highly specific and allow for the identification of bacteria within their habitat (Schwieger and Tebbe, 1998; Stackebrandt et al., 1991).

We found that a strain with the signature of *A. pretiosum* is likely to be present in the rhizosphere and possibly also within the endorhiza of the *P. verrucosa* plant.

2.2.1. Investigation of the V3 variable site of the 16S rDNA

The DNA was isolated from bacteria of the rhizosphere of three different *P. verrucosa* plants. The first plant (annotated FG1 or plant "V" in Pullen et al., 2003) was known to accumulate maytansine at a concentration of 342 μg/g root (dry weight). According to our hypothesis, this plant should have a root-associated maytansine producing bacterium. The bacteria of two additional plants of unknown or no maytansine (**1**) content were also analyzed. As reference strains, three groups of bacteria were employed. The first group (*K. putterlickiae*, R12, R27a) comprised those strains which we had previously isolated from the rhizosphere of a *P. verrucosa* plant using conventional techniques (see Section 2.1 and Groth et al., 2003). The second group consisted of those bacteria (*A. pretiosum* ssp. *pretiosum* (DSM 44132), *A. pretiosum* ssp. *auranticum* (ATCC 1565), *A. mirum* (DSM 43827)) which could be identical to or closely related to the maytansine producing soil bacterium. *Amicolatopsis mediterranei* S699 (G. Lancini, Lepetit Research Laboratory, Geranzano, Italy) and *Actinoplanes teichomyceticus* (DSM 43866) strain were included as a third group. The V3 region (302 bp) was chosen as the first target for those oligonucleotides (F-243/R-513_P) which are specific for actinomycetes (Table 1). Thus, the DNA amplified should be actinomycetous 16S rDNA only. After amplification by PCR, the phosphorylated DNA strand was digested and the remaining strands analyzed by SSCP (Fig. 2).

The gel (Fig. 2) shows that the SSCP technique differentiates between the different actinomycetous bacteria. Three bands of the FG1 plant (lane 7) are most interesting. Migration of band 2 in lane 7 matched a band of the authentic *A. pretiosum* ssp. *auranticum* strain in lane 4. After isolating and amplifying band 2 (lane 7), three clones were collected for sequencing. A data bank analysis indicated that one clone was likely to stem from *Streptomyces nodosus* while one was identical to *A. pretiosum* ssp. *auranticum*. The third clone had a 99% homology to *A. pretiosum* ssp. *auranticum*. Band 1 in lane 4 (reference bacterium *A. pretiosum* ssp. *auranticum*) was also investigated and two clones were isolated which showed a 99% homology to *A. pretiosum* ssp. *auranticum*. It is not to be expected that rDNA sequences from a given eubacterium are identical in every case. rDNA genes are arranged in multiple copies and may be subject to a limited amount of sequence heterogeneities (Nübel et al., 1996).

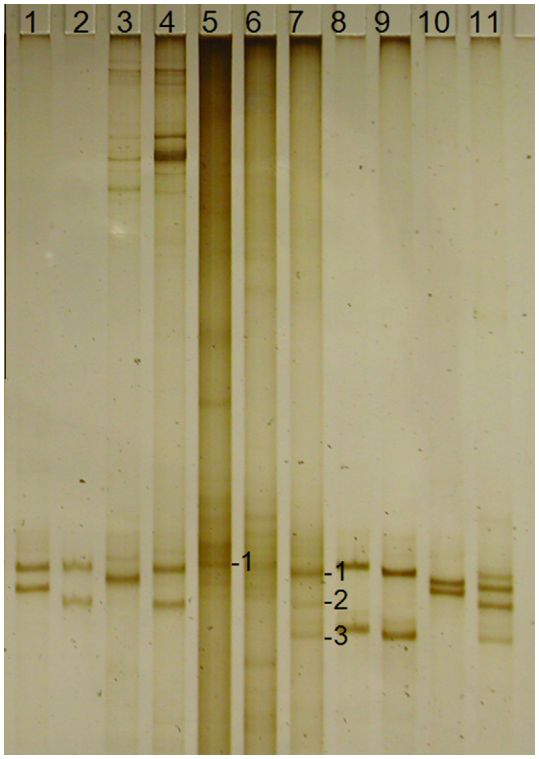

Fig. 2. SSCP gel of the V3 region of the 16S rDNA of the bacterial community in the rhizosphere of three different *P. verrucosa* plants (lane 5 and 6 of unknown maytansine content, lane 7 of known maytansine content i.e. plant FG1 also annotated plant "V" in Pullen et al., 2003). Reference strains: *Kitasatospora putterlickiae* (lane 1), *Actinosynnema mirum* (lane 2), *Actinosynnema pretiosum* ssp. *pretiosum* (lane 3), *Actinosynnema pretiosum* ssp. *auranticum* (lane 4), R12 (lane 8), 27a (lane 9), *Amycolatopsis mediterranei* (lane 10), *Actinoplanes teichomyceticus* (lane 11).

We conclude that:

(i) a known bacterial species (in this case *A. pretiosum* ssp. *auranticum*) taken through a SSCP procedure followed by sequencing can be recovered by this technique.
(ii) a bacterium with a V3 region identical to that of *A. pretiosum* ssp. *auranticum* is present in the rhizosphere of *P. verrucosa*.

We would like to point out, however, that a differentiation between the three *Actinosynnema* strains (*A. pretiosum* ssp. *pretiosum*, *A. pretiosum* ssp. *auranticum*, *A. mirum*) may be difficult when using the V3 region (compare lanes 2 and 4 in Fig. 2). We have also sequenced the same V3 region of *A. pretiosum* ssp. *auranticum*, however, using oligonucleotides F-27 and R-1492 (Weisburg et al., 1991) which gave a DNA stretch spanning not 302 but 1439 base pairs. The sequence was submitted to the NCBI (accession: DQ464900) and compared to those of *A. pretiosum* ssp. *pretiosum* (accession: AF114800) and *A. mirum* (accession: D85475). The

Table 1
Oligonucleotides used in polymerase chain reactions and sequencing of variable regions V3–V9 of the 16S rDNA.

Target region	V3	V3–V9	V4 and V5	V6–V9
Oligo-nucleotides	F-243 R-513_P	F-243 R-1470	Com-1 Com-2_P	F-984 R-1378_P
References	Heuer et al. (1997)	Heuer et al. (1997) Weisburg et al. (1991)	Lane et al. (1985)	Nübel et al. (1996)
Base pairs of rDNA targeted	226–243 513–528	226–243 1470–1492	519–536 907–926	968–984 1378–1402
Length (bp) of PCR products	302	1266	407	434

sequence of the V3 region of *A. pretiosum* ssp. *auranticum* had a 99% homology to that of *A. mirum* and a 98% homology to *A. pretiosum* ssp. *pretiosum*. The sequence of the V3 region discerns the genus *Actinosynnema* from other actinomycetes used in this experiment but does not differentiate between our three *Actinosynnema* reference strains. Because of the high homology between these strains we analyzed additional regions of the rDNA.

2.2.2. Investigation of the V4 and V5 variable site of the rDNA

In the next attempt to throw light on the possible presence of a maytansine producing bacterium in the rhizosphere, we employed oligonucleotides F-243/R1492 targeting an extended DNA stretch with a sequence characteristic of actinomycetes. The resulting PCR product had a length of 1266 base pairs and comprised regions V4 and V5. "Nested" oligonucleotides Com-1 and Com-2_P were subsequently used to amplify a 407 base pair stretch out of the first PCR product in preparation of a SSCP analysis. This time not only the rhizosphere but also the endorhiza were investigated. As reference organisms again *A. pretiosum* ssp. *auranticum*, R12, R27a, and *K. putterlickiae* were analyzed in parallel. The DNA of each reference organism showed its own characteristic migration in the SSCP (gel not shown). The DNA of the endorhiza and the rhizosphere of the FG1 (*P. verrucosa*) plant showed a band with the same migration as the DNA from *A. pretiosum* ssp. *auranticum*. The band from the rhizosphere of FG1 was sequenced. Among 4 clones isolated during preparation of the sequencing process three showed a 99–100% homology to that of *A. pretiosum* ssp. *auranticum*. This is additional proof for the presence of this bacterium in the rhizosphere of *P. verrucosa.* The gel showed also that the bacterium very likely is also present in the endorhiza of this plant.

2.2.3. Investigation of the V6–V9 variable site of the rDNA

According to Stackebrandt et al. (1991), the V6 region is particularly suitable to differentiate between *Streptomyces* species. Two ways to analyze the V6 and V9 region were used.

(i) Oligonucleotides F-984/R-1378_P were employed in a PCR reaction which directly gave a 434 base pair stretch of the V6 region.
(ii) This same DNA sequence was also obtained in a second (indirect) approach in which first the bacterial DNA typical of actinomycetes was amplified (oligonucleotides F-243/R-1492) and then the resulting DNA used for a nested PCR with oligonucleotides F-984/R-1378_P. This gave the same DNA sequence also with a length of 434 baise pairs although enriched when compared to the sample obtained in the first and direct approach.

Comparison of authentic strains R27a, R12, *K. putterlickiae*, and *A. pretiosum* ssp. *auranticum* by SSCP and gel electrophoresis showed that these bacterial strains are clearly discernible. The DNA from the rhizosphere of *P. verrucosa* gave identical bands in the direct and the indirect approach mentioned above. Both bands perfectly matched a band comigrating with that of the authentic *A. pretiosum* ssp. *auranticum*. We conclude again that *A. pretiosum* ssp. *auranticum* is likely to be present in the rhizosphere of the *P. verrucosa* plant.

2.2.4. SSCP analysis of the 16S–23S internal transscribed spacer (ITS)

The genomes of many procaryotes contain several copies of different rrn operons comprising 16S rDNA, the internal transcribed spacer (ITS), 23S rDNA and 5S rDNA. The internal transcribed spacer is a DNA region highly variable in length and sequence (Embley and Stackebrandt, 1994; Scheinert et al., 1996; Stackebrandt et al., 1991). To analyze the region between 16S rDNA and 23S rDNA oligonucleotides F-1525-1542 and R-33-48-P (Zhang et al., 1997) were employed. The forward oligonucleotide (F-1525-1542) is located at the end of the 16S rDNA, the reverse phosphorylated oligonucleotide at the beginning of the 23S rDNA gene. The DNA between both oligonucleotides spans ca 300 base pairs. Amplification products of DNA from the bacterial rhizosphere of the *P. verrucosa* plant FG1 (i.e. plant V in Pullen et al., 2003) and the *A. pretiosum* ssp. *auranticum* reference strain were checked by agarose gel for a DNA sequence comprising ca 300 base pairs. The 300 bp sequence was found by conventional gel electrophoresis to be present. Subsequently the remaining bulk DNA was separated by SSCP. The 300 base pair stretch consisting of a single strand molecule was isolated from the gel and amplified using the same oligonucleotides as above. In this case, however, the reverse oligonucleotide was not phosphorylated. The resulting DNA stretch was prepared for sequencing. Five clones were selected. Two of these clones showed 99% homology to the sequence of the reference strain *A. pretiosum* ssp. *auranticum* (DQ459053). Thus, it was again evident that this strain is likely to be present in the rhizosphere of *P. verrucosa*.

3. Discussion

The techniques employed in this study (SSCP combined with rDNA sequencing) have widely been used in the characterisation of the bacterial community of the plant rhizosphere (Guertler and Stanisich, 1996; Scheinert et al., 1996; Schwieger and Tebbe, 1998).

The reliability and reproducibility of these techniques used here are evident from the fact that previously isolated bacterial strains R12, R27a and *K. putterlickiae* are not only detectable in the rhizosphere of *P. verrucosa* by culture-dependent but also by culture-independent (i.e. molecular biological) techniques. This, and the detection of different rDNA sequences unique to *A. pretiosum*, are the basis for the conclusion that a maytansine producing *A. pretiosum* strain very likely is present in the rhizosphere and the endorhiza. The bacterial strain is likely to be adapted to its rhizosomal biotope and not necessarily identical in all aspects to *A. pretiosum* ssp. *auranticum* deposited in a bacterial culture collection (ATCC 31565). Our observations show, however, that a bacterium at least very closely related to *A. pretiosum* ssp. *auranticum* is present in the rhizosphere of the *P. verrucosa* plant. The presence of this bacterial strain suggests – but does not yet prove – that the maytansine in *P. verrucosa* is unlikely to be a biosynthetic product of the plant, but very likely synthesized by an *A. pretiosum* strain. This conclusion is also in agreement with the observation that antibiotic synthesizing microorganisms are frequent inhabitants of a plant's rhizosphere (Berg et al., 2005, 2007; Whipps, 2001). These bacteria protect the plant from attack by plant pathogenic microorganisms. Indeed, the rhizosphere of a plant is a microbial "hot spot" with an intense interaction between microorganisms and between microorganisms and plants. This biotope forms a nutrient-rich niche for microorganisms as a result of exudation of compounds from the root system.

The presence of maytansine in *P. verrucosa* plants is confined to the root system and the lower part of the branches of the plants (Pullen et al., 2003). The tips of twigs and branches are devoid of maytansine suggesting that its supply to the plant proceeds through the root system.

There are different ways how microorganisms infect a plant. Plants belonging to the family *Convolvulaceae* contain ergoline alkaloids which are produced by plant-associated clavicipitaceous fungi (Leistner and Steiner, 2009; Markert et al., 2008; Steiner et al., 2011; Steiner and Leistner, 2011). The fungi are seed transmitted to the following plant generation. This kind of transmission is called vertical transmission.

A horizontal transmission is likely to occur in plants of the *Celastraceae* such as *P. verrucosa.* As outlined above (see Section 2.2), we favor the idea that a bacterium capable to synthesize maytansine infects the root system of *P. verrucosa* (Cassady et al., 2004; Pullen et al., 2003). Since the infection process is likely to occur repeatedly and *de novo* for every single plant, the occurrence of maytansine (**1**) is observed in some individual plants while others remain unaffected and are devoid of maytansine (**1**). We reported previously on two *Putterlickia* plants (plants V and W in Pullen et al., 2003) characterized by the same developmental stage growing at the same biotope with one containing maytansine [700 μg/g dried wood from branches (Pullen et al., 2003), 342 μg/g dried root material (see Section 2.2.1)], whereas in the other one ansa antibiotics were not detectable. The conclusion that the source of maytansine in *P. verrucosa* and other ansa antibiotic containing plants (*Euphorbiaceae* and *Rhamnaceae*) is a bacterium (Cassady et al., 2004; Pullen et al., 2003) is also based on the observation that in general most ansa antibiotics are bacterial products (Lancini, 1986). The genetic machinery required to synthesize ansa antibiotics is without doubt located in *A. pretiosum* (maytansine and its congeners) (Cassady et al., 2004) and *Amycolatopsis mediterranei* (rifamycin and its congeners) (Floss, 2006) but not in the respective host plants (compare Section 2.1).

3.1. Concluding remarks

The chemical structures of bacterial and plant ansamitocins are identical as far as the basic ansamitocin skeleton is concerned, but individual compounds in both organisms differ in their substitution patterns. Among 22 known plant ansamitocins (maytansinoids) only two occur also in bacteria while 20 are exclusively plant metabolites. Among 20 bacterial ansamitocins, 18 occur exclusively in bacteria, (Cassady et al., 2004). This raises the question whether the host plants contribute to the structural diversity of plant borne maytansinoids. The fact that ansamitocins occurring in the plant families *Euphorbiaceae* and *Rhamnaceae* represent even structures not observed in *Celastraceae* shows again that the possible contribution of host plants to the structural diversity of ansamitocins is an important but unsettled question.

4. Experimental

4.1. Plant material

P. verrucosa (E. Meyer ex Sonder) Szyszyl. plants were collected in South Africa, cultivated in the green house at the Institute für Pharmazeutische Biologie in Bonn, Germany, and voucher specimens deposited at the H.G.W.J. Schweickert-Herbarium of the Botanical Institute Pretoria in South Africa and in Bonn, Germany, as described in Pullen et al. (2003).

4.2. Identification and quantitative determination of maytansine by high resolution LC-MS

Air-dried roots (2 g) of *P. verrucosa* were separated by cutting and was extracted with MeOH (10 ml × 2) at room temperature for 24 h and 8 h, respectively. The resulting extract was analyzed by means of high resolution mass spectrometry. The spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 200–1000) with nominal mass resolving power of 60000 at m/z 400 with a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard; Bis(2-ethylhexyl)phthalate : m/z = 391.284286. The spectrometer was attached with an Agilent (Santa Clara, USA) 1200 HPLC system consisting of a LC-pump, PDA detector (λ = 280 nm), autosampler (injection volume 10 μl) and column oven (30 °C). Following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260 °C, tube lens 70 V. N_2 was used as sheath gas (50 arbitrary units) and auxiliary gas (5 arbitrary units). He served as the collision gas. The separations were performed by using a Nucleodur Sphinx column (50 × 3 mm,1.8 μm particle size) from Macherey–Nagel (Düren, Germany) with a H_2O (+0.1% HCOOH) (A)/CH_3CN (+0.1% HCOOH) (B) gradient (flow rate 300 μl min^{-1}). Samples were analyzed by using a gradient program as follows: 70% A isocratic for 2 min, linear gradient to 70% B over 8 min, after 100% B isocratic for 5 min, the system returned to its initial condition (70% A) within 0.5 min, and was equilibrated for 4.5 min. Maytansine occurred at a retention time of 7.8 min. The value obtained from the high resolution spectra for this compound (692.29364 u) was in good agreement with the proposed elemental composition of the $[M + H]^+$ ion $C_{34}H_{47}O_{10}N_3Cl$ (theoretical mass: 692.29445 u, error 1.16 ppm). In addition, the compound was identified and quantitatively determined by comparison of the spectroscopic and chromatographic data using an authentic reference substance obtained from the National Institute of Health, USA.

4.3. Isolation of culturable bacteria from the rhizosphere of P. verrucosa

Culturable bacteria (R12 and R27a) were isolated from roots of the maytansine containing plant FG1 (also annotated plant "V", Pullen et al., 2003) which had been brought to Bonn from its natural habitat in South Africa. The plant was kept in soil of its South African biotope in the green house in Bonn. Hairy roots were removed from the plant using germ free tweezers and the fraction of adhering bacterial cells isolated as described by Schwieger and Tebbe, 1998. The bacterial suspension was streaked onto agar plates containing the R2A agar medium (Difco BD, Heidelberg, Germany). Single cell colonies were isolated using the R2A as well as the Nutrient Agar of Oxoid, Wesel, Germany. The isolated bacteria were screened for the presence of an AHBA synthase gene as described by Pullen et al. (2003).

4.4. Isolation of DNA from the bacterial community of the rhizosphere and the endorhiza of P. verrucosa

DNA was isolated from the bacterial cell suspension (i.e. the rhizoshere) and the endorhiza following the procedures of Schwieger and Tebbe, 1998. DNA from cells of the endorhiza was isolated from the same roots that were used for the collection of bacteria from the rhizosphere.

4.5. Standard PCR reaction

The PCR mix contained the following components: DNA (50 pg to 50 ng/50 μl), oligomers (50 pmol/μl, 1.0 μl each), dNTPs (25 mM, 1 μl each), buffer as supplied with the polymerase (10-fold concentrated, 5 μl), $MgCl_2$ (25 mM, up to 4 μl), DMSO (2.5–5 μl) (only employed for a GC rich DNA), DNA polymerase (HotMasterTaq-Polymerase, Eppendorf, Hamburg, Germany) (1–2.5 U), H_2O ad 50 μl.

4.5.1. Temperature protocol for 16S rDNA (oligonucleotides F-27, R-1492) (Weisburg et al., 1991)

Denaturing (95 °C, 5 min); annealing (42 °C, 1 min); 30 cycles extension (72 °C, 2 min), denaturing (95 °C, 2 min), annealing (42 °C, 0.5 min); extension (72 °C, 20 min); 4 °C.

4.5.2. Temperature protocol for the 16S rDNA-23S rDNA spacer

Oligonucleotides were chosen to match the end of the 16s (1525–1542F) and the beginning of the 23S rDNA gene (33–48R, *Escherichia coli* numbering). 25 Cycles consisting of denaturing (95 °C, 40 s), annealing (52 °C, 30 s), extension (72 °C, 20 s), 4 °C (Zhang et al., 1997).

4.6. PCR reactions for DNA from bacterial communities and SSCP experiments

The PCR mix contained the following components: DNA (50 ng/50 μl), oligomers (50 pmol/μl, 1 μl each), dNTPs (25 mM, 1 μl each), buffer as supplied with the polymerase (10-fold concentrated, 5 μl), $MgCl_2$ (25 mM, 6 μl), DMSO (2.5 μl), T4 gene 32 protein according to Schwieger and Tebbe (1998) (1 μl at a concentration of 5 μg/ml), Expand High Fidelity Taq-Polymerase mix (3.75 U) (Roche Diagnostics, Mannheim, Germany) water ad 50 μl.

4.6.1. Temperature protocol for the V3 region of actinomycetes (oligonucleotides F-243, R-513_P or R-1378_P)

Denaturing (94 °C, 5 min); annealing (63 °C, 1 min); 10 cycles extension (72 °C, 2 min), denaturing (94 °C, 1 min), annealing (63 °C, 1 min); 25 cycles extension (72 °C, 2 min) (to each single extension 5 s are added), denaturing (94 °C, 1 min), annealing (63 °C, 1 s); extension (72 °C, 10 min), 4 °C.

4.6.2. Temperature protocol for regions V4 and V5 (oligonucleotides Com-1, Com-2P)

Denaturing (94 °C, 3 min); annealing (50 °C, 1 min); 10 cycles extension (72 °C, 1.5 min), denaturing (94 °C, 1 min), annealing (50 °C, 1 min); 25 cycles extension (72 °C, 1 min) (to each single extension 5 s are added), denaturing (95 °C, 1 min), annealing (70 °C, 40 s); extension (74 °C, 7 min), 4 °C.

4.6.3. Temperature protocol for V6–V9 regions of actinomycetes (oligonucleotides F-243, R-1492 and F-984, R-1378_P)

Denaturing (95 °C, 5 min); annealing (53 °C, 1 min); 10 cycles extension (72 °C, 1.5 s), denaturing (94 °C, 1 min), annealing (53 °C, 1 min); 25 cycles extension (72 °C, 1.5 s) (to each single extension 5 s were added), denaturing (94 °C, 1 min), annealing (53 °C, 1 min); extension (72 °C, 7 min), 4 °C.

The same temperature protocol was employed for the amplification of regions V6 and V9 in a "nested" PCR using oligonucleotides F-984 and R-1378_P except that the annealing temperature was set to 50 °C instead of 53 °C.

4.7. SSCP analysis of partial 16S rDNA genes

This was carried out as described by Schwieger and Tebbe, 1998. After SSCP bands were silver stained and extracted from the gel reamplification and sequencing was possible. As noted previously (Schwieger and Tebbe, 1998) a further SSCP run occasionally gave additional bands. It was assumed that DNA strands formed additional conformations.

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