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Isolation, Diversity, and Antimicrobial Activity of Rare Actinobacteria from Medicinal Plants of Tropical Rain Forests in Xishuangbanna, China▽†

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Endophytic actinobacteria are relatively unexplored as potential sources of novel species and novel natural products for medical and commercial exploitation. Xishuangbanna is recognized throughout the world for its diverse flora, especially the rain forest plants, many of which have indigenous pharmaceutical histories. However, little is known about the endophytic actinobacteria of this tropical area. In this work, we studied the diversity of actinobacteria isolated from medicinal plants collected from tropical rain forests in Xishuangbanna. By the use of different selective isolation media and methods, a total of 2,174 actinobacteria were isolated. Forty-six isolates were selected on the basis of their morphologies on different media and were further characterized by 16S rRNA gene sequencing. The results showed an unexpected level of diversity, with 32 different genera. To our knowledge, this is the first report describing the isolation of *Saccharopolyspora*, *Dietzia*, *Blastococcus*, *Dactylosporangium*, *Promicromonospora*, *Oerskovia*, *Actinocorallia*, and *Jiangella* species from endophytic environments. At least 19 isolates are considered novel taxa by our current research. In addition, all 46 isolates were tested for antimicrobial activity and were screened for the presence of genes encoding polyketide synthetases and nonribosomal peptide synthetases. The results confirm that the medicinal plants of Xishuangbanna represent an extremely rich reservoir for the isolation of a significant diversity of actinobacteria, including novel species, that are potential sources for the discovery of biologically active compounds.

The class *Actinobacteria* accounts for a high proportion of soil microbial biomass and contains the most economically significant prokaryotes, producing more than half of the bioactive compounds in a literature survey (46), including antibiotics (6), immunosuppressive agents (55), antitumor agents (18), and enzymes (64). Actinobacteria belonging to the genus *Streptomyces*, in particular, are excellent producers. The emergence of drug resistance in many bacterial pathogens and the current increase in the number of fungal infections has caused a resurgence of interest in finding new reserves of biologically active compounds (63). As the search for novel natural products continues, it becomes apparent that the rate of discovery of new compounds from soil streptomycetes has decreased, whereas the rate of reisolation of known compounds has increased (28). Recently, evidence has accumulated that rare actinomycete species, which are often very difficult to isolate and cultivate, might represent a unique source of novel biologically active compounds (4). On the other hand, new microbial habitats need to be examined in the search for novel bioactive compounds. One biologically important but relatively overlooked niche is the inner tissues of higher plants. Early studies have demonstrated that some actinobacteria can form intimate associations with plants and colonize their inner tissues. *Frankia* species and *Streptomyces scabies* can penetrate their hosts and establish either pathogenic or endophytic associations (5, 24). The actinomycetes that reside in the tissues of living plants and do not visibly harm the plants are known as endophytic actinobacteria (37). These actinobacteria are relatively unstudied and are potential sources of novel natural products for exploitation in medicine, agriculture, and industry (73).

Endophytic actinobacteria have attracted attention in recent years, with increasing reports of isolates from a range of plant types, including crop plants (cereals, such as wheat and rice, as well as potatoes, carrots, tomatoes, and citrus) (2, 16, 62, 71, 74, 80) and medicinal plants (75, 88). The culturable endophytic actinobacteria from these plants were found to fall within a narrow species distribution: *Streptomyces* spp. were the predominant species, and *Microbispora*, *Micromonospora*, *Nocardioides*, *Nocardia*, and *Streptosporangium* were the common genera. Endophytic actinobacteria have been demonstrated to improve and promote the growth of host plants as well as to reduce disease symptoms caused by plant pathogens through various mechanisms, including the production of secondary metabolites, which are used in direct antagonism against pests and diseases (9, 10, 12), changes in host physiology (42), and the induction of systemic acquired resistance in plants (15). Another significant function found for these actinobacteria was antibiotic activity, suggesting that endophytic actinobacteria can be an interesting source for bioprospecting.

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New antibiotics from endophytic *Streptomyces* spp.—alnumycin, munumbicins A to D, and coronamycins—have been reported (7, 11). Recently, two novel antitumor anthraquinones, lupinacidins A and B, were isolated from a new endophytic *Micromonospora* sp. (43). Moreover, new species of endophytic actinobacteria have been increasingly reported (25, 35). Thus, endophytic actinobacteria are expected to be potential sources of new species and new bioactive agents.

Of the myriad ecosystems on earth, those with the greatest general biodiversity seem also to have the greatest number and the greatest diversity of endophytes (73). Tropical and temperate rain forests are the most biologically diverse terrestrial ecosystems on earth and thus the greatest possible resource for the acquisition of novel microorganisms and their products (73). One area of enormous plant biodiversity is Xishuangbanna, located in the People's Republic of China at the border with Myanmar. This area lies at the ecotone between the Asian tropics and subtropics and is dominated by tropical seasonal rain forests (87). Xishuangbanna contains more than 5,000 species of vascular plants, comprising 16% of China's total plant diversity, and more than 3,000 are endemic species (53, 60), many of which have ethnobotanical histories. Until the present, little research was carried out to isolate endophytic actinobacteria and their secondary metabolites from Xishuangbanna (36, 86). In our long-term study of endophytic actinobacterial diversity and bioactive metabolites from tropical rain forest medicinal plants in Xishuangbanna, many bioactive endophytic *Streptomyces* spp. have been isolated (49). However, the work to date is insufficient to provide a general understanding of the diversity, distribution, and ecology of tropical rain forest endophytic actinobacteria and to facilitate further exploitation of the diverse functions of this novel microbial source.

In the present study, the diversity of rare endophytic actinobacteria associated with medicinal plants from the tropical rain forest in Xishuangbanna was investigated by combining special culturing techniques. The selected isolates were also identified by 16S rRNA gene analysis. The overall aims of this study were (i) to analyze the actinobacterial community and reveal whether the rain forest investigated in Xishuangbanna represents a valuable source for abundant endophytic actinobacteria and new species, (ii) to evaluate the antimicrobial activities of these actinobacteria and the biosynthetic potential of related secondary metabolites, and (iii) to study the relationships between the taxa of these endophytic actinobacteria and the isolation methods applied.

MATERIALS AND METHODS

Sample collection. An extensive collection of various plant materials was set up in the Menglun Nature Reserve (21°56′N, 101°11′S) and the Jinghong Nature Reserve (22°01′ to 22°19′N, 100°47′ to 100°57′S) in the tropical seasonal rain forest in Xishuangbanna for 2 years (from November 2005 to May 2006 and from September 2006 to June 2007). The selection of each plant for endophyte isolation was based on its local ethnobotanical properties, including its antibacterial, insecticidal, antitumor, and wound-healing properties. No history indicates that the plants had ever been studied for endophytic actinobacteria previously. Healthy root, stem, and leaf samples of each plant were placed in sterile plastic bags, taken to the laboratory, and subjected to isolation procedures within 96 h. Some representative plants of the nearly 90 selected for study are *Phyllanthus urinaria*, *Kadsura heteroclita*, *Maesa indica*, *Rauvolfia verticillata*, *Paris yunnanensis*, *Maytenus austroyunnanensis*, *Gloriosa superba*, *Scoparia dulcis*, *Tadehagi triquetrum*, *Goniothalamus* sp., *Cephalotaxus* sp., and *Azadirachta* sp.

Selective isolation procedures and media. Samples were air dried for 48 h at room temperature and were then washed with an ultrasonic step (160 W, 15 min) to remove the surface soils and adherent epiphytes completely. After drying, the samples were subjected to a five-step surface sterilization procedure: a 4- to 10-min wash in 5% NaOCl, followed by a 10-min wash in 2.5% $Na_2S_2O_3$, a 5-min wash in 75% ethanol, a wash in sterile water, and a final rinse in 10% $NaHCO_3$ for 10 min. After being thoroughly dried under sterile conditions, the surface-sterilized tissues were subjected to continuous drying at 100°C for 15 min. Surface-treated tissues were then pretreated by one of the following methods.

Method 1. Most of the samples were aseptically crumbled into small fragments, directly placed on the selective medium, and incubated at 28°C for 2 to 8 weeks.

Method 2. Some samples (1.0 g), treated as described above, were mixed in a mortar with 0.5 g of sterile powdered calcium carbonate and then placed in a petri dish. Two milliliters of sterilized tap water was added to the samples to create a moist environment. The petri dish was maintained at 28°C for 2 weeks and was then air dried at room temperature to a constant weight. Parts of the samples were directly diluted to 10^{-4} with sterile water and were spread-plated onto medium. The other mixed samples were continuously enriched by the method of Otoguro et al. (65). Samples were placed in a glass vessel and flooded with 50 ml of 10 mmol phosphate buffer (pH 7.0) containing 10% plant extract or soil extract at 28°C for 5 h to liberate actinomycete spores. A portion (10 ml) of the flooding mixture was transferred to a centrifuge tube and centrifuged at 1,500 × *g* for 30 min. After settling for 30 min, a portion of the supernatant enriched with spores was serially diluted with sterile tap water and plated onto medium.

Method 3. Some plants, e.g., *Maytenus austroyunnanensis*, were aseptically crumbled into small fragments and isolated using the combined enzymatic hydrolysis and differential centrifugation method (44). The homogenate was centrifuged at 200 × *g* for 20 min at 4°C, and the supernatant was subsequently centrifuged at 3,000 × *g* for 30 min at 4°C to collect the sediments, which were diluted to 10^{-2} with sterile water and spread-plated onto medium.

Pretreated dilutions of the samples (200 μl) were spread over the surfaces of solid media designed for the cultivation of actinobacteria (Table 1). The inoculated plates were incubated at 28°C for 2 to 4 weeks. The pHs of all media were adjusted to 7.0 to 7.4. All media were amended with nalidixic acid (50 mg liter^{-1}) and nystatin (100 mg liter^{-1}) to suppress the growth of gram-negative bacteria and fungi. As the colonies appeared from the plates, they were observed and selected carefully according to their characteristics. Special attention was given to the non-mycelium-forming actinobacteria. They were inoculated onto yeast extract-malt extract agar (ISP 2) slants and incubated at 28°C for 2 to 3 weeks.

Effectiveness of surface sterilization. Two experiments were carried out to check the effectiveness of the sterilization procedures. First, the surface-sterilized tissue was imprinted onto ISP 2 agar, incubated at 28°C, and then checked for microbial growth. Second, the surface-sterilized samples were washed in sterile distilled water three times, soaked in 5 ml sterile water, and stirred for 1 min. A 0.2-ml aliquot of the suspension was then inoculated onto ISP 2 agar plates, incubated at 28°C, and observed for microbial growth. If no microbial growth occurred on the surface of the medium, the sterilization was considered complete.

Preliminary identification of actinobacteria. Isolates were tentatively grouped and dereplicated by observing their morphological and cultural characteristics, including the characteristics of colonies on plates and slants, the presence of aerial mycelia and substrate mycelia, spore mass color, distinctive reverse colony color, diffusible pigment, and sporophore and spore chain morphology. Many colonies that were similar in color, shape, and size were observed for hyphal length and structure with light microscopes, which allowed them to be segregated into distinct isolates. The cell wall type was also determined on the basis of the occurrence of isomers of diaminopimelic acid in order to distinguish the streptomycetes from other spore-forming actinomycetes, since whole-organism hydrolysates of *Streptomyces* strains contain the LL isomer and other spore-forming actinomycetes contain *meso*-diaminopimelic acid. The diagnostic sugars of the representatives of each group were also detected (38). Based on the preliminary grouping, 46 isolates were selected for further research.

DNA extraction, sequencing, and analysis. The 46 isolates selected were subjected to 16S rRNA gene sequence analysis for precise genus and species identification. The identities of the organisms were determined based on partial or nearly full length 16S rRNA gene sequence analysis. The genomic DNA of each isolate was extracted using a method of Li et al. (52). The 16S rRNA genes from pure cultures were amplified using the primer pair PA–PB (Table 2). PCR was carried out under the following conditions: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. The reaction mixture (50 μl) contained de-

TABLE 1. Compositions of the 11 different media used for the isolation of endophytic actinobacteria from plant samples

Medium	Composition	Reference
M1	TWYE	19
M2	Modified TWYE supplemented with plant extract	This study
M3	Glycerol-asparagine agar (ISP 5)	72
M4	HV agar	40
M5	Inorganic salts-starch agar (ISP 4)	72
M6	YIM 38 medium (4.0 g malt extract, 4.0 g yeast extract, 4.0 g glucose, vitamin mixture, 15.0 g agar)	This study
M7	Raffinose-histidine agar	83
M8	Sodium propionate agar (1.0 g sodium propionate, 0.2 g L-asparagine, 0.9 g KH_2PO_4, 0.6 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 15.0 g agar)	This study
M9	Cellulose-proline agar (2.5 g cellulose, 2.0 g sodium pyruvate, 0.25 g KNO_3, 1.0 g proline, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g K_2HPO_4, 0.5 g $CaCl_2$, 10 mg $FeSO_4 \cdot 7H_2O$, 15.0 g agar)	This study
M10	Trehalose-proline medium [5.0 g fucose, 1.0 g proline, 1.0 g $(NH_4)_2SO_4$, 1.0 g NaCl, 2.0 g $CaCl_2$, 1.0 g K_2HPO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 15.0 g agar]	This study
M11	Xylan-arginine agar [2.5 g xylan, 1.0 g arginine, 1.0 g $(NH_4)_2SO_4$, 2.0 g $CaCl_2$, 1.0 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, and 15.0 g agar]	This study

oxynucleoside triphosphates (0.25 mM each), 1× reaction buffer (20 mM Tris [pH 8.4], 50 mM KCl), 3 mM $MgCl_2$, 1 μM each primer, 1.25 U of *Taq* DNA polymerase, and 1 ng of template DNA. (All PCR reagents were purchased from TaKaRa, Dalian, China.) The PCR products were separated by agarose gel electrophoresis, purified using QIAquick gel extraction kits (Qiagen, Hilden, Germany), and sequenced on an ABI Prism 3730 sequencer.

The 16S rRNA gene sequences determined were compared with the GenBank/EMBL/DDBJ databases by using the BLASTN (1) search program. A phylogenetic tree was constructed by the neighbor-joining (70) method using the MEGA software package, version 3.1 (45), after pairwise alignments using the CLUSTAL_X program, version 1.8 (79). The stability of relationships was assessed by performing bootstrap analyses (27) of the neighbor-joining data based on 1,000 resamplings.

Detection of PKS-I, PKS-II, and NRPS sequences. Three sets of degenerate primers for the amplification of genes encoding polyketide synthases I and II (PKS-I and PKS-II) and nonribosomal peptide synthetases (NRPS) from the isolates tested were carried out as recommended by Ayuso-Sacido and Genilloud (3) and Metsä-Ketelä et al. (58) (Table 2). The reaction mixture contained 2.5 U of *Taq* DNA polymerase, 1 mM $MgCl_2$, 0.4 mM deoxynucleoside triphosphates, 2 μM each primer, and 5% dimethyl sulfoxide in a 50-μl reaction volume. Control reaction mixtures had no actinobacterial DNA template. Thermocycling conditions consisted of one denaturation step of 94°C for 5 min; 30 amplification cycles of 94°C for 1 min, 57°C (for K1F–M6R and A3F–A7R) or 58°C (for KS_α–KS_β) for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min.

Fermentation, extraction, and evaluation of antimicrobial activity. Each isolate was cultured in soybean mannitol liquid medium (12 g soybean flour and 20 g mannitol in 1,000 ml tap water [pH 7.2 to 7.4]) at 28°C and was shaken at 180 rpm). After 7 to 12 days of cultivation, the fermentation broth was extracted with ethanol. Each organic solvent extract was then evaporated under reduced pressure to yield an ethanol extract. The ethanol extracts were then used for antimicrobial activity screening. The inhibitory effect of the extract obtained from endophytic actinobacteria was tested using the paper disk (diameter, 7 mm) assay method (57). A 25-μl volume of the ethanol extract suspension was pipetted onto each disk and incubated at 37°C, and the diameters of the inhibition zones were measured after 24 h. A 25-μl volume of ethanol was used as the control. The pathogenic bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* and the pathogenic yeast *Candida albicans* were used as indicator organisms for the determination of antimicrobial activity. The pathogenic microorganisms were deposited in the Yunnan Institute of Microbiology, Yunnan University.

Nucleotide sequence accession numbers. The DNA sequences of the 46 isolates have been deposited in GenBank under the accession numbers given in Table 3.

RESULTS AND DISCUSSION

Evaluation of surface sterilization protocol. Surface sterilization is important for studying endophytes. The imprinted agar on an ISP 2 plate from each surface-sterilized sample showed no microbial growth after 15 days of incubation at 28°C. In addition, ISP 2 agar plates spread with the last water from the washing of plant samples also failed to grow colonies of microorganisms after 2 weeks of incubation. This indicated that the five-step surface sterilization protocol was effective at killing the epiphytic microorganisms. Thus, the subsequent isolates can be considered true endophytic actinobacteria.

In this study, a 2.5% sodium thiosulfate solution was used instead of sterilized water rinses to remove the chlorine cover left on the plant surfaces by hypochlorite disinfection. This procedure was based on the consideration that even though plant tissues were rinsed thoroughly with sterilized water after treatment with NaOCl, toxic compounds were still present on their surfaces, and these compounds could kill endophytes or at least stress them so much that they would be unable to form colonies on the plates. By our tentative experiments, the in-

TABLE 2. PCR primers used in this study

Primer name	Sequence (5′–3′)	Target gene	Length of target gene fragment (bp)	Reference
PA	5′-AGAGTTTGATCCTGGCTCAG-3′	16S rRNA	1,400–1,500	20
PB	5′-AAGGAGGTGATCCAGCCGCA-3′			
K1F	5′-TSAAGTCSAACATCGGBCA-3′	PKS-I	1,200–1,400	3
M6R	5′-CGCAGGTTSCSGTACCAGTA-3′			
KS_α	5′-TSGCSTGCTTGGAYGCSATC-3′	PKS-II	600	58
KS_β	5′-TGG AANCCG CCGAABCCTCT-3′			
A3F	5′-GCSTACSYSATSTACACSTCSGG-3′	NRPS	700–800	3
A7R	5′-SASGTCVCCSGTSCGGTAS-3′			

TABLE 3. Actinobacteria isolated from different organs of host plants using three different methods, with the taxonomic statuses of plants and similarity values for 16S rRNA gene sequences

Isolate (GenBank accession no.)	Host plant		Organ	Closest cultivated species (GenBank accession no.)	Similarity (%)	Isolation method[a]
	Genus and species	Family				
YIM 61095 (EU814512)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Saccharopolyspora flava* AS 4.1520^T (AF154128)	97.7	1
YIM 65359 (FJ214364)	*Tripterygium hypoglaucum*	*Celastraceae*	Stem	*Saccharopolyspora flava* AS 4.1520^T (AF154128)	98.4	1
YIM 60513 (EU005371)	*Gloriosa superba*	*Liliaceae*	Stem	*Saccharopolyspora gregorii* NCIB 12823^T (X76962)	99.1	1
YIM 65085 (FJ214357)	*Osyris wightiana*	*Santalaceae*	Root	*Amycolatopsis pretoriensis* NRRL B-24133^T (AY183356)	98.0	3
YIM 65117 (FJ214358)	*Ficus tikoua*	*Moraceae*	Root	*Lentzea albida* IFO 16102^T (AB006176)	99.1	1
YIM 61043 (FJ214340)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Pseudonocardia zijingensis* JCM 11117^T (AF325725)	98.6	3
YIM 56051 (EU200680)	*Callicarpa longifolia*	*Verbenaceae*	Leaf	*Pseudonocardia alni* IMSNU 20049^T (AJ252823)	97.5	1
YIM 56035 (DQ887489)	*Lobelia clavata*	*Campanulaceae*	Leaf	*Pseudonocardia kongjuensis* DSM 44525^T (AJ252833)	98.4	1
YIM 65003 (EU325542)	*Cercidiphyllum japonicum*	*Cercidiphyllaceae*	Leaf	*Rhodococcus fascians* DSM 20669^T (X79186)	99.6	1
YIM 61441 (FJ214349)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Nocardia carnea* DSM 46071^T (AF430037)	98.3	3
YIM 61394 (FJ214346)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Tsukamurella tyrosinosolvens* DSM 44234^T (AY238514)	99.9	2
YIM 65220 (FJ214359)	*Paris yunnanensis*	*Liliaceae*	Leaf	*Mycobacterium monacense* B9-21-178^T (AF107039)	97.2	3
YIM 65001 (EU375845)	*Schima* sp.	*Theaceae*	Stem	*Dietzia maris* DSM 43672^T (X79290)	99.8	1
YIM 65002 (EU375846)	*Cercidiphyllum japonicum*	*Cercidiphyllaceae*	Root	*Dietzia natronolimnaea* 15LN1^T (X92157)	99.5	1
YIM 61527 (FJ214354)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Gordonia sputi* DSM 43896^T (X80634)	99.0	3
YIM 61401 (FJ214347)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Gordonia polyisoprenivorans* W8130^T (DQ385609)	97.9	3
YIM 60475 (EU200683)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Streptomyces hainanensis* YIM 47672^T (AM398645)	99.2	1
YIM 65188 (EU925562)	*Sedum* sp.	*Crassulaceae*	Root	*Streptomyces specialis* GW 41-1564^T (AM934703)	97.5	1
YIM 65287 (FJ214361)	*Tripterygium wilfordii*	*Celastraceae*	Root	*Blastococcus aggregatus* DSM 4725^T (AJ430193)	99.6	1
YIM 61359 (FJ214343)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Polymorphospora rubra* DSM 44947^T (AB223089)	97.6	2
YIM 65312 (FJ214363)	*Tripterygium wilfordii*	*Celastraceae*	Root	*Dactylosporangium aurantiacum* DSM 43157^T (X93191)	98.0	2
YIM 65268 (FJ214360)	*Berberis chingii*	*Berberidaceae*	Stem	*Catellatospora chokoriensis* 2-25(1)^T (AB200231)	99.0	1
YIM 61481 (FJ214350)	*Maytenus austroyunnanensis*	*Celastraceae*	Leaf	*Micromonospora narashino* DSM 43172^T (X92602)	99.3	3
YIM 61363 (FJ214344)	*Maytenus austroyunnanensis*	*Celastraceae*	Leaf	*Micromonospora peucetia* DSM 43363^T (X92603)	98.0	1
YIM 65293 (FJ214362)	*Tripterygium wilfordii*	*Celastraceae*	Stem	*Kineosporia aurantiaca* IFO 14067^T (D86937)	98.2	1
YIM 65377 (FJ214365)	*Tripterygium wilfordii*	*Celastraceae*	Stem	*Kineococcus aurantiacus* IFO 15268^T (X77958)	98.0	2
YIM 61515 (FJ214352)	*Maytenus austroyunnanensis*	*Celastraceae*	Leaf	*Promicromonospora aerolata* IFO 16526^T (AJ487303)	99.0	3
YIM 56864 (EU200679)	*Cymbopogon citratus*	*Poaceae*	Root	*Promicromonospora sukumoe* DSM 44121^T (AJ272024)	99.4	2
YIM 60535 (EU200684)	*Ginkgo* sp.	*Ginkgoaceae*	Leaf	*Oerskovia jenensis* DSM 46097^T (AJ314850)	99.3	1
YIM 61525 (FJ214353)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Janibacter melonis* KCTC 9987^T (AY522568)	99.8	1
YIM 61410 (FJ214348)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Microbacterium paraoxydans* H110b^T (EF204432)	100	3

Continued on following page

TABLE 3—*Continued*

Isolate (GenBank accession no.)	Host plant		Organ	Closest cultivated species (GenBank accession no.)	Similarity (%)	Isolation method[a]
	Genus and species	Family				
YIM 61510 (FJ214351)	*Maytenus austroyunnanensis*	*Celastraceae*	Leaf	*Arthrobacter mysorens* LMG 16219^T (AJ639831)	98.2	3
YIM 65004 (FJ214355)	*Polyspora axillaris*	*Theaceae*	Root	*Micrococcus flavus* CGMCC 1.5361^T (DQ491453)	99.7	1
YIM 56238 (EU005372)	*Aquilaria sinensis*	*Thymelaeaceae*	Root	*Micrococcus flavus* CGMCC 1.5361^T (DQ491453)	99.0	1
YIM 61341 (FJ214342)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Nocardiopsis dassonvillei* JPL-3^T (AY030320)	98.4	1
YIM 61333 (FJ214341)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Nocardiopsis dassonvillei* JPL-3^T (AY030320)	98.2	3
YIM 56155 (FJ214339)	*Duranta repens* Linn	*Verbenaceae*	Root	*Actinocorallia aurantiaca* JCM 8201^T (AF134066)	99.1	2
YIM 60542 (EU005370)	*Millettia reticulata*	*Papilionaceae*	Leaf	*Actinocorallia herbida* IFO 15485^T (D85473)	99.1	2
YIM 61435 (FJ157185)	*Maytenus austroyunnanensis*	*Celastraceae*	Leaf	*Actinomadura atramentaria* DSM 43919^T (AJ420138)	97.4	2
YIM 61375 (FJ214345)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Streptosporangium vulgare* DSM 44112^T (X89957)	97.4	2
YIM 61105 (FJ157184)	*Maytenus austroyunnanensis*	*Celastraceae*	Leaf	*Nonomuraea candida* DSM 45086^T (DQ285421)	98.2	1
YIM 65070 (FJ214356)	*Osyris wightiana*	*Santalaceae*	Stem	*Herbidospora cretacea* IFO 15474^T (D85485)	99.5	2
YIM 61503 (FJ157186)	*Maytenus austroyunnanensis*	*Celastraceae*	Stem	*Jiangella alkaliphila* DSM 45079^T (AM422451)	98.8	2
YIM 56134 (EU200681)	*Carex baccans* Nees	*Cyperaceae*	Root	*Glycomyces algeriensis* NRRL B-16327^T (AY462044)	98.9	1
YIM 56256 (EU200682)	*Scoparia dulcis*	*Scrophulariaceae*	Root	*Glycomyces sambucus* DSM 45047^T (DQ460469)	99.7	1
YIM 61331 (EU814511)	*Maytenus austroyunnanensis*	*Celastraceae*	Root	*Glycomyces sambucus* DSM 45047^T (DQ460469)	98.7	1

[a] In method 1, samples were aseptically crumbled into small fragments, placed directly on the selective medium, and incubated at 28°C for 2 to 8 weeks. Method 2, combined calcium carbonate enrichment with RC. Method 3, combined enzymatic hydrolysis and the differential centrifugation method.

crease in the number of CFU over that for the control was obvious (see Table S1 in the supplemental material). This was consistent with the effects of sterilization of rice seed surfaces with 2.0% sodium thiosulfate instead of water rinses (59) and with the fact that thiosulfate can suppress the detrimental effects of hypochlorite on skin (34). It is also known that an alkaline environment favors the growth of actinomycetes but not that of endophytic fungi. The growth of fungal endophytes was effectively inhibited by soaking the plant tissues in a 10% $NaHCO_3$ solution in our study so that the actinobacterial endophytes could grow out of tissues before fungi overgrew the samples and masked the actinobacteria. The present surface sterilization method was effective at helping us acquire endophytic actinobacteria.

Selective isolation of culturable endophytic actinobacteria from medicinal plants. On the basis of characteristic colonial morphology, notably the ability to form aerial hyphae and substrate mycelia, organisms putatively identified as actinobacteria were selected. In total, 2,174 actinobacterial strains were isolated from nearly 90 selected plant samples. According to the preliminary morphological identification, the most abundant genus was *Streptomyces* (87%), a finding consistent with other reports from different hosts (9, 16, 82). Since the aim of our study was to isolate rare non-*Streptomyces* actinobacterial genera, 280 strains that did not seem to belong to the genus *Streptomyces* according to the morphological criteria, or whose classification was in doubt, were further checked for cultural and micromorphological characteristics and were examined for the presence of isomers of diaminopimelic acid. Based on these results, *Pseudonocardia* was the dominant genus (57%), followed by *Nocardiopsis* (8.3%), *Micromonospora* (6.4%), *Streptosporangium* (3.8%), and other organisms (24.5%) that could not be accurately identified to the genus level. The genera *Micromonospora*, *Streptosporangium*, and *Nocardiopsis* have also been reported frequently from a wide range of host plants, such as maize, lichens, and wheat (21, 30, 33). Forty-six strains, including representatives of each group, were subjected to 16S rRNA gene sequence analysis.

We used several isolation procedures in this study in order to obtain as many endophytic actinobacteria as possible. Compared to a pretreatment method commonly used previously for isolating endophytic actinobacteria (cutting samples into small pieces about 0.2 by 0.5 cm [10, 82]), method 1 enlarged the colonization area of samples on the agar plates, which could be helpful for recovering endophytes.

We also used the combined calcium carbonate enrichment–rehydration and centrifugation (RC) pretreatment procedure developed earlier (method 2). This method allowed efficient isolation of *Dactylosporangium*, *Kineosporia*, *Kineococcus*, *Herbidospora*, *Jiangella*. and *Promicromonospora* species. Although

at present the precise mechanism of the calcium carbonate effect is not clear, previous work has shown that after the isolation source is mixed with powdered calcium carbonate, its pH is altered in favor of the growth of actinomycete propagules (81) and that calcium ions have the ability to stimulate the formation of aerial mycelia by several actinomycete cultures (61). Previous isolation of soil samples with calcium carbonate has demonstrated significant increases in the relative plate counts of the actinomycete population (81). The centrifugation stage eliminated streptomycetes and other nonmotile actinomycetes from the liquid phase, thereby facilitating the selective growth of rare, especially motile actinomycetes on the isolation plates subsequent to inoculation (39). By the combined calcium carbonate–RC procedures, Otoguro et al. (65) have successfully isolated diverse *Actinokineospora* spp. and other actinomycetes from soils and plant litter. The present study demonstrated that the enrichment stage with calcium carbonate and the RC procedure was also suitable for the isolation of zoosporic and other rare actinobacteria from endophytic habitats.

Pretreatment method 3 was especially useful for isolating endophytic actinobacteria. Besides the predominant *Streptomyces*, *Pseudonocardia*, *Nocardiopsis*, and *Micromonospora* species, species of other genera, including *Amycolatopsis*, *Nocardia*, *Nonomuraea*, *Actinomadura*, *Gordonia*, *Promicromonospora*, and *Mycobacterium*, were also recovered. This procedure was based on the fact that endophytic bacteria reside mainly in intercellular spaces in plant tissues, and only rarely in intracellular spaces and inside vascular tissues (78). By mild treatment with enzymes hydrolyzing plant cell walls and subsequent differential centrifugation, most of the microbes associated with plant tissues were collected, and the microbial cells were maintained intact, which could be demonstrated by scanning electron microscopy (23). Centrifugation is commonly used to collect the intercellular (apoplastic) fluid of plant tissue (8, 22) and also has been successfully applied for the isolation of endophytic bacteria from sugarcane stems by a force of 3,000 $\times$ *g* (23). This method appears to be time-consuming, requiring both surface sterilization and centrifugation, but several samples can be centrifuged at the same time. It was first successfully applied to the isolation of diverse endophytic actinobacteria from culture in our study. Recently, Wang et al. (84) reported on the diversity of uncultured plant-associated microbes obtained by an improved DNA extraction method and increased differential centrifugation at a force of 5,000 $\times$ *g*. Actinobacteria were the dominant microbial group, accounting for 37.7% of the 16S rRNA gene library, which also demonstrated the feasibility of using pretreatment method 3 for the isolation of endophytic actinobacteria.

Actinobacteria were isolated on all of the 11 selective isolation media. Observations on the frequency of isolation by method 1 suggested that most strains were isolated from the relatively simple nutrient media, such as TWYE, sodium propionate agar, and HV, which were most effective for the isolation of endophytic actinobacteria. Other media seemed to be less suitable, due to the fact that their high nutrient concentrations allowed fast-growing bacteria to overgrow slower-growing actinobacteria. This finding was consistent with the results of Coombs and Franco (16). It is interesting that the ISP 5 agar, raffinose-histidine agar, trehalose-proline agar, and the sodium propionate, cellulose-proline, and xylan-arginine media that we designed are more effective for isolation by procedures 2 and 3. Much higher total numbers of colonies formed on these media than on others (data not shown). Girousse et al. (32) have detected asparagine as the main amino acid (70%) in the phloem sap of alfalfa. Additionally, Mazzafera and Goncalves (56) found that in the xylem sap of coffee, asparagine and glutamine accounted for almost 90% of the nitrogen in the form of amino acids. Similar results were reported by Tejera et al. (77): the predominant nitrogen compounds in the apoplastic sap of sugarcane stems were amino acids (50 to 70% N), including serine, proline, alanine, and aspartic acid. These findings provided a rationale for attempting to use amino acids as nitrogen sources for isolation from culture; nevertheless, the real principle of isolation and the role of these substances in relation to the establishment of the symbiosis of endophytes require further experimental analysis.

Diversity of endophytic actinobacteria by 16S rRNA gene analysis. The endophytic actinobacteria isolated in this study displayed considerable diversity. They were distributed among 10 suborders—*Pseudonocardineae* (eight strains), *Corynebacterineae* (eight strains), *Streptomycineae* (two strains), *Frankineae* (one strain), *Micromonosporineae* (five strains), *Kineosporiineae* (two strains), *Micrococcineae* (eight strains), *Streptosporangineae* (eight strains), *Propionibacterineae* (one strain), and *Glycomycineae* (three strains)—within the class *Actinobacteria* on the phylogenetic tree (see Fig. S1 in the supplemental material), including 32 genera and more than 40 species. Notably, some uncommon genera from the endophytic environment, such as *Amycolatopsis*, *Lentzea*, *Pseudonocardia*, *Rhodococcus*, *Nocardia*, *Tsukamurella*, *Mycobacterium*, *Dietzia*, *Gordonia*, *Dactylosporangium*, *Kineosporia*, *Janibacter*, *Kineococcus*, *Arthrobacter*, *Micrococcus*, *Nonomuraea*, *Herbidospora*, and *Glycomyces*, were discovered in the present study. The percentages of 16S rRNA gene sequence similarities (97.2 to 100%) of these isolates to the closest type strains are presented in Table 3. As shown in Table 4, 34 out of the 46 strains were isolated from woody plants, and most of the 46 strains were from roots (20 isolates) and stems (14 isolates), although the detailed taxa of strains had no relationship to the taxonomic affiliations (species, genera, families) of the host plants.

Further phylogenetic analyses of some of the isolates are shown in Fig. S2a to S2e in the supplemental material. Pairwise comparison of the 16S rRNA gene sequences from the three *Pseudonocardia* isolates (YIM 56035, YIM 56051, and YIM 61043) showed relatively high similarities (98.4, 97.5, and 98.6%) to the type strains of *Pseudonocardia kongjuensis*, *Pseudonocardia antarctica*, and *Pseudonocardia zijingensis*. However, the phylogenetic analysis indicated that these isolates were diversely distributed within this genus and clustered singly in the phylogenetic tree (see Fig. S2a in the supplemental material), suggesting that isolates YIM 56035, YIM 56051, and YIM 61043 might belong to new species. The *Saccharopolyspora* isolates (YIM 65359, YIM 61095, and YIM 60513) had lineages that were distinct from each other and from other members of the genus; they formed distinct subclades in the tree supported by high bootstrap values (see Fig. S2b in the supplemental material). Strain YIM 61095 has been characterized as a new species of this genus (67). The 16S rRNA gene sequences of YIM 65359 and YIM 60513 showed 98.4% and

TABLE 4. Statistical analyses of the relationships between the taxa of the 46 endophytic actinobacterial strains and the organs and types of plant samples

Plant	Organ	No. of isolates	Isolate taxon		
			Suborder	Family	Genus
Herbaceous[a]	Root	7	*Pseudonocardineae* *Streptomycineae* *Micrococcineae* *Streptosporangineae* *Glycomycineae*	*Pseudonocardiaceae* *Streptomycetaceae* *Promicromonosporaceae* *Thermomonosporaceae* *Glycomycetaceae*	*Amycolatopsis* *Lentzea* *Glycomyces* *Pseudonocardia* *Streptomyces* *Promicromonospora* *Actinocorallia*
	Stem	2	*Pseudonocardineae* *Streptosporangineae*	*Pseudonocardiaceae* *Streptosporangiaceae*	*Saccharopolyspora* *Herbidospora*
	Leaf	3	*Pseudonocardineae* *Corynebacterineae*	*Pseudonocardiaceae* *Mycobacteriaceae*	*Pseudonocardia* *Mycobacterium*
Woody[b]	Root	13	*Pseudonocardineae* *Streptomycineae* *Corynebacterineae* *Frankineae* *Micromonosporineae* *Micrococcineae* *Streptosporangineae* *Glycomycineae*	*Pseudonocardiaceae* *Streptomycetaceae* *Dietziaceae* *Nocardiaceae* *Geodermatophilaceae* *Micromonosporaceae* *Intrasporangiaceae* *Micrococcaceae* *Nocardiopsaceae* *Glycomycetaceae*	*Saccharopolyspora* *Pseudonocardia* *Dietzia* *Glycomyces* *Streptomyces* *Nocardia* *Janibacter* *Blastococcus* *Dactylosporangium* *Micrococcus* *Nocardiopsis* "*Plantactinospora*"
	Stem	12	*Pseudonocardineae* *Corynebacterineae* *Micromonosporineae* *Kineosporiineae* *Micrococcineae* *Streptosporangineae* *Propionibacterineae*	*Pseudonocardiaceae* *Tsukamurellaceae* *Dietziaceae* *Nocardiaceae* *Micromonosporaceae* *Kineosporiaceae* *Microbacteriaceae* *Nocardiopsaceae* *Streptosporangiaceae* *Nocardioidaceae*	*Saccharopolyspora* *Tsukamurella* *Dietzia* *Jiangella* *Gordonia* *Catellatospora* *Kineosporia* *Kineococcus* *Microbacterium* *Nocardiopsis* *Streptosporangium*
	Leaf	9	*Corynebacterineae* *Streptosporangineae* *Micrococcineae* *Micromonosporineae*	*Nocardiaceae* *Streptosporangiaceae* *Thermomonosporaceae* *Cellulomonadaceae* *Promicromonosporaceae* *Micromonosporaceae* *Micrococcaceae*	*Rhodococcus* *Nonomuraea* *Actinomadura* *Actinocorallia* *Oerskovia* *Promicromonospora* *Micromonospora* *Arthrobacter*

[a] A total of 12 isolates, representing 6 suborders, 7 families, and 10 genera, were from herbaceous plants.
[b] A total of 34 isolates, representing 10 suborders, 18 families, and 28 genera, were from woody plants.

99.1% identities to the nearest neighbors, YIM 61095 and *Saccharopolyspora gregorii*, respectively. DNA-DNA hybridizations, phenotypic comparisons, and chemotaxonomic analysis classified YIM 65359 and YIM 60513 as new species of the genus *Saccharopolyspora*. The *Glycomyces* isolates (YIM 56134, YIM 61331, and YIM 56256) formed distinct clusters within the *Glycomyces* 16S rRNA gene tree (see Fig. S2c in the supplemental material), although they showed the highest 16S rRNA gene similarities—98.9, 98.7, and 99.7%—to the closest recognized strains. Each of these three isolates has been identified as a different new species of the genus *Glycomyces* (66, 68). Although the 16S rRNA gene sequences of two isolates, YIM 61515 and 56864, showed as much as 99.0% and 99.4% identity to the related type strains of the genus *Promicromonospora* (see Fig. S2d in the supplemental material), DNA-DNA relatedness values (unpublished data) were well below the 70% cutoff point recommended by Wayne et al. (85) for the delineation of strains that belong to the same genomic species, indicating that they are still new species. It was particularly interesting to recover isolate YIM 61359. Comparison of the 16S rRNA gene sequences of this isolate and its closest neighbors indicated that YIM 61359 was closely related to the type strains of the genera *Micromonospora* (96.6% to 98.1% similarity) and *Salinispora* (97.7% similarity) and to that of *Polymorphospora rubra* (97.6% similarity). However, YIM 61359 formed a distinct monophyletic clade within the family *Micromonosporaceae* (see Fig. S2e in the supplemental material) in the phylogenetic tree, most closely related to the first new obligately marine actinomycete genus, *Salinispora*, which produces the antibiotic salinosporamide A (26, 54). This indicated

TABLE 5. Antimicrobial activities and PKS/NRPS genes of culturable actinobacteria from medicinal plants

Isolate no.	Activity[a] against:				Presence[b] of gene		
	Candida albicans	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	PKS-I	PKS-II	NRPS
YIM 61095	−	−	−	−	−	−	+
YIM 65359	−	−	−	−	−	−	−
YIM 60513	−	−	−	+	+	−	−
YIM 65085	−	−	−	−	−	−	−
YIM 65117	−	++	++	−	−	−	+
YIM 61043	−	+	−	−	−	−	+
YIM 56051	−	++	−	−	−	+	−
YIM 56035	−	−	−	−	−	+	−
YIM 65003	−	−	−	−	−	−	+
YIM 61441	−	−	−	−	+	−	+
YIM 61394	−	−	−	−	−	−	−
YIM 65220	−	+	+	−	−	+	−
YIM 65001	−	−	−	−	−	−	−
YIM 65002	−	−	−	−	−	−	−
YIM 61527	+	−	+	−	−	−	−
YIM 61401	−	−	−	−	−	−	−
YIM 60475	−	−	++	−	−	−	−
YIM 65188	−	−	−	−	−	−	−
YIM 65287	−	−	−	−	−	−	−
YIM 61359	−	++	−	++	−	−	−
YIM 65312	−	++	−	−	−	−	−
YIM 65268	−	−	++	−	−	+	−
YIM 61481	++	−	−	−	−	−	+
YIM 61363	++	−	−	−	+	−	−
YIM 65293	−	−	+	−	−	+	+
YIM 65377	−	−	−	−	−	+	+
YIM 61515	−	+	++	++	−	−	−
YIM 56864	−	−	−	++	−	−	−
YIM 60535	−	−	−	−	−	−	−
YIM 61525	−	−	−	−	−	−	−
YIM 61410	+	−	−	−	−	−	−
YIM 61510	−	−	−	−	−	−	−
YIM 65004	−	−	−	−	−	−	−
YIM 56238	−	−	−	−	−	−	−
YIM 61341	+++	−	++	−	−	−	+
YIM 61333	+++	−	++	+++	−	−	+
YIM 56155	−	+	−	−	−	−	−
YIM 60542	−	−	−	−	−	−	−
YIM 61435	−	−	−	−	−	−	−
YIM 61375	++	−	−	−	+	+	+
YIM 61105	+++	−	−	−	−	−	−
YIM 65070	−	−	++	−	−	−	+
YIM 61503	−	−	++	−	−	−	−
YIM 56134	−	+++	+	−	+	−	−
YIM 56256	−	−	−	−	−	−	−
YIM 61331	−	−	−	−	−	−	−

[a] Estimated by measuring the diameter of the clear zone of growth inhibition. Symbols: −, no activity; +, ++, and +++, weak activity, moderate activity, and strong activity, respectively.

[b] +, present; −, absent.

that isolate YIM 61359 should represent a new genus, proposed as "*Plantactinospora* gen. nov.," of the family *Micromonosporaceae* and that it merits research on secondary metabolites. Although the 16S rRNA gene sequences of isolates YIM 65003 (*Rhodococcus*), YIM 65001 (*Dietzia*), YIM 65002 (*Dietzia*), YIM 60475 (*Streptomyces*), YIM 65188 (*Streptomyces*), YIM 56238 (*Micrococcus*), and YIM 61503 (*Jiangella*) showed more than 97% similarity to those of the nearest type strains, they have already been identified as new species (13, 14, 48, 50, 51, 69) by polyphasic taxonomic analyses. Detailed characterizations of other isolates in comparison with the nearest type strains are currently being carried out in order to determine

whether they are new species. Taxonomic descriptions of these putative novel species will be given elsewhere.

We isolated and cultured 46 representatives of 32 genera of actinobacteria from medicinal plants. This is the first study to recover such a high diversity of culturable actinobacteria from endophytic environments. In addition, to our knowledge, this is the first time that strains of the rarely recovered genera *Saccharopolyspora*, *Dietzia*, *Blastococcus*, *Dactylosporangium*, *Promicromonospora*, *Oerskovia*, *Actinocorallia*, and *Jiangella* have been isolated and cultured from the inner parts of plants. Taechowisan et al. (75) isolated 330 isolates belonging to four different genera (*Streptomyces*, *Microbispora*, *Nocardia*, and *Micromonospora*) from 36 medicinal plant species from Thailand. Tan et al. (76) recovered 619 actinomycetes from different cultivars of tomato, and all of them were *Streptomyces* spp. Recently, Lee et al. (47) identified eight endophytic actinobacterial genera for only 81 isolates of Chinese cabbage roots; *Microbispora* spp. (67%) were the most common isolates, followed by *Streptomyces* spp. (12%) and *Micromonospora* spp. (11%). Obviously, the richness and diversity of actinobacteria isolated from the tropical rain forest in Xishuangbanna in our present study are much higher than those for other reports, and tropical rain forests have potential as excellent sources of actinobacteria and new species. Therefore, it seems clear that endophytic communities are diverse and that while the extent of diversity may differ between different sample collection regions and different plant species, the diversity of actinobacteria isolated was largely dependent on the isolation method.

Antimicrobial activity and detection of PKS and NRPS genes in the selected actinobacteria. All 46 isolates were tested for antimicrobial activities against the pathogenic bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* and against the pathogenic yeast *Candida albicans*. Twenty-four of the 46 isolates (52.2%) exhibited activity against at least one of the pathogenic microorganisms tested. Activity against *B. subtilis* was clearly the most frequent (12 isolates [26.1%]). Activity against *P. aeruginosa* was the least frequent (10.9%), while 19.6% and 17.4% of the isolates were active against *S. aureus* and *C. albicans*, respectively. Six isolates were found to inhibit two pathogens. Two isolates, YIM 61515 and YIM 61333, appeared to have a broad spectrum of antimicrobial activity. Four isolates, YIM 61341, YIM 61333, YIM 61105, and YIM 56134, exhibited relatively strong inhibitory effects against the pathogenic microorganisms *C. albicans*, *S. aureus*, and *P. aeruginosa*. The strong inhibitory activities of these strains against a variety of pathogens suggested that these endophytic actinobacteria may be potential candidates for the production of bioactive compounds. Some of the strains are currently being chemically analyzed in order to identify active compounds and to assess their novelty.

The 46 strains were screened for the presence of PKS-I, PKS-II, and NRPS sequences by specific amplification of chromosomal DNA with primer sets K1F–M6R, KS_{α}–KS_{β}, and A3F–A7R, respectively (Table 5), to evaluate their biosynthetic potential in terms of natural product drug discovery. NRPS sequences were detected in 12 isolates (26.1%), whereas PKS-I and PKS-II sequences were detected in only 5 and 7 of the 46 strains (10.9% and 15.2%), respectively. Strains YIM 65293 and YIM 65377 gave positive amplification products with both the PKS-II and NRPS primers. Both PKS-I and NRPS gene sequences were amplified from strain YIM 61441. All three target genes were detected in strain YIM 61375. Several representative amplified PKS-I, PKS-II, and NRPS gene products were cloned and sequenced, and subsequent analysis confirmed that they indeed encode parts of the expected biosynthetic enzymes (see accession no. FJ615259).

The antimicrobial activity results and the detection of functional genes seemed to have no direct correlation in the present study. Several isolates, such as YIM 61095 and YIM 65377, had no antimicrobial activity, but target genes could be successfully amplified. For 28 strains (60.9%), the PKS and NRPS genes were not detected using the selected degenerate primer pairs, which have been used in a broad survey of these genes in a similar study (3). The absence of amplification products from some of the strains may reflect the lack of PKS-I, PKS-II, and NRPS genes, though it is also possible that these specific degenerate primer pairs might not be suitable for amplifying these genes. Furthermore, not all NRPS genes are involved in the biosynthesis of bioactive secondary metabolites; indeed, the products of such genes may be involved in functions such as iron metabolism or quorum sensing (29). It is also possible that the genes detected by PCR are nonfunctional. Nevertheless, prescreening of isolates with PCR primers targeting genes encoding the biosynthesis of bioactive compounds is an effective approach for detecting novel and useful secondary metabolites (17, 31, 41, 58).

Conclusions. In summary, this study demonstrates that an unexpected diversity of actinobacteria colonize the interiors of medicinal plants in Xishuangbanna tropical rain forests, and it suggests that these strains might represent a valuable source of new species and biologically active compounds with antimicrobial activity and genes for their biosynthesis. The isolation procedure is a critically important step in studies of endophytic actinobacteria. It is necessary to improve traditional selective isolation methods in order to recover the untapped majority of endophytic actinobacteria. Although many questions remain regarding the ecological function of actinobacteria in the endophytic environment, as well as their evolution and biogeographic distribution, the increasing numbers of rare actinobacteria isolated from medicinal plants indicate that these plants are potentially unique sources of novel actinobacteria with promising potential to produce highly bioactive metabolites. That potential should not be overlooked.

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