Bioactive endophytic streptomycetes from the Malay Peninsula

Noraziah M. Zin1, Nurul I. M. Sarmin2, Norazli Ghadin1, Dayang F. Basri1, Nik M. Sidik2, W. M. Hess3 & Gary A. Strobel4

1Department of Biomedical Sciences, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia; 2School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Malaysia; 3Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT, USA; and 4Department of Plant Sciences, Montana State University, Bozeman, MT, USA

Correspondence: Gary A. Strobel, Department of Plant Sciences, Montana State University, Bozeman, MT 59717, USA; Tel.: +1 406 994 5148; fax: +1 406 994 760; e-mail: uplgs@montana.edu

Received 18 April 2007; revised 18 May 2007; accepted 19 May 2007. First published online 30 June 2007.

DOI:10.1111/j.1574-6968.2007.00819.x

Editor: Aharon Oren

Keywords
streptomycetes; antifungal agents; endophytes; DNA; rRNA.

Abstract

Three novel endophytic streptomycetes have been isolated and characterized from plants with ethnobotanical uses on the Malay Peninsula including: Hottea grandiflora (family – Aristolochiaceae), Polyalthia spp. (family – Annonaceae), and Mapania sp. (family – Cyperaceae). Each isolate, as studied by scanning electron microscopy, has small hyphae, and produces typical barrel-shaped spores arising by hyphal fragmentation. Interestingly, although none has any detectable antibacterial killing properties, each has demonstrable killing activity against one or more pathogenic fungi including organisms such as Phytophthora erythroseptica, Pythium ultimum, Sclerotinia sclerotiorum, Mycosphaerella fijiensis and Rhizoctonia solani. Molecular biological studies on the rRNA gene sequence of each isolate revealed that it is distinct from all other genetic accessions of streptomycetes in GenBank, and each bears some genetic similarity to other streptomycetes. The bioactivity of each microbe was extractable in various organic solvents.

Introduction

As a group, the streptomycetes are an extremely valuable group of microorganisms to mankind because they have provided nearly 80% of the world’s antibiotics and literally all of these microorganisms have come from the world’s soils (Arai, 1976). The advent of drug resistance in many bacterial pathogens, the current increase in the number of fungal infections and a need for more environmentally compatible controls for agricultural pests and pathogens has caused a resurgence of interest in finding other reserves of biologically active compounds (NIAID, 2001). It is now obvious that these filamentous bacteria also exist as endophytes within the living tissues of certain higher plants (Bieber et al., 1998; Castillo et al., 2003, 2005, 2006; Ezra et al., 2004). Because the majority of them is novel, it has been suggested that they may also serve as sources of novel bioactive compounds (Ezra et al., 2004; Castillo et al., 2005, 2006, 2007). A search for plants hosting such endophytic streptomycetes has now begun (Strobel & Daisy, 2003).

Because the plant kingdom is so large, over 300,000 species, a selection of which plants to study for endophytic streptomycetes also might be based on an ethnobotanical approach. That is, the possibility that any healing properties of the plant used by indigenous peoples may in fact be related to the presence of one or more endophytes within the tissues of the plant (Strobel & Daisy, 2003). Also, because endophytic streptomycetes are associated with a eukaryotic organism, the possibility of them making products that are not toxic to its host organism seems more likely. Thus, one of the major concerns in drug discovery, relating to the toxicity of a drug candidate, may be averted by dealing with endophytic streptomycetes and their biologically active products.

Many recently isolated endophytic streptomycetes possess unique rRNA gene sequences, many make totally novel biologically active products, many possess unique hyphal structures, while others have interesting and totally novel cultural characteristics (Ezra et al., 2004; Castillo et al., 2005, 2006, 2007). Also, previously, plants representing families such as Fagaceae, that were present during the era of the ancient Gondwanaland, yielded numerous novel bioactive endophytic streptomycetes (Castillo et al., 2007). Thus, it appears extremely likely that still other biologically active streptomycete isolates exist in plants throughout the world.

An area of enormous plant biodiversity is Malaysia, with over 15,000 plant species, and specifically there are over 9000 on the Malay Peninsula alone, with 3–4500 being endemic (Mittermeier et al., 1999). However, in this study, a concerted attempt was made to collect and study plants that
strictly have an ethnobotanical background; that is, they possess attributes of such things as healing wounds, curing fevers, and reducing skin infections. Until this time, however, no concerted search for endophytic streptomycetes had ever been carried out on the Malay Peninsula in spite of the fact that it is one of the most biological diverse places on the earth.

Ultimately, genetically unique endophytic streptomycetes having a range of biological activities were isolated from representatives of three plant families associated with the Malay Peninsula and partially characterized. This report describes the details on the isolation and bioactivities of these unique endophytic streptomycetes from the peninsula and shows that some of the bioactive products are extractable with organic solvents.

Materials and methods

Selection of plants and location of collection points

Plants (stem segments and some root materials) were collected from four different locations in peninsular Malaysia. The representative areas were: northern area of Sungai Sedim, Kedah; the west coast – Sungai Tekala, Selangor; the east coast–Bukit Bauk, Terengganu; and the southern part of peninsular Malaysia – Gunung Panti, Johor. Each plant selected for endophyte isolation was based on its local ethnobotanical properties including its properties to heal wounds, cure fevers, and/or skin infections. No history exists indicating that the plants had ever been previously studied for endophytic microorganisms.

Each plant was tagged, stored in a clean plastic bag, and given a voucher specimen number that was entered in the botanical collection of the Universiti Kebangsaan, Malaysia. Some representative plants, of the nearly 200 selected for study, selected for study are as follows: *Thottea grandiflora* (family – Aristolochiaceae), *Artabotrys* sp., *Fissistigma kingii*, *Goniothalamus ridleyi*, *Cinnamomum sp.*, *Chassalia sp.*, *Barringtonia reticulata*, *Brucea javanica*, *Mapania* sp., *Psychotria* sp., *Etingera sp.*, *Helicia* sp., *Labisia pumula*, *Elettariopsis* sp., *Aglaiia* sp., *Pentagramma* sp., *Polyalthia* sp. (family – Annonaceae), *Mapania* sp. (family – Cyperaceae), *Senna alata*, *Phyllanthus amarus*, and *Melastoma* sp.

Isolating and storing Streptomycete spp.

Both representative root and stem plant samples were subjected to surface treatment procedures to eliminate surface contaminating microbial communities which included a wash in 70% alcohol, followed by exposure to 95% ethanol followed by immersion in 0.9% NaOCl and drying. The bark or epidermis was aseptically removed and the tissues lying beneath were excised and placed onto an agar surface [starch casein agar (SCA)] containing cyclohexamide (50 mg L\(^{-1}\)). As the microorganisms appeared during the course of several weeks, they were carefully picked and re-cultured as a means of checking for cultural purity. At least three organisms appearing as actinomycetes, yielding an earthy odor in culture, seemed as likely candidates as potential streptomycetes. They were cultured on nutrient agar (NA) and stored in 15% glycerol solution at –70 °C. They were kept in the living microbe collection at Montana State University as cultures MSU 2337-HTBP-SUK 03 (from *Polyalthia* spp.); MSU 2338-HBB-SUK 01 (from *T. grandiflora*); and MSU 2339-SSP-SUK 02 (from *Mapania* spp.).

Scanning electron microscopy (SEM)

Fruiting structures of the microorganisms appearing on carnation leaves or on agar plates (NA) were examined by stereo and light microscopy and then in a more detailed fashion by SEM. These colonies and structures were fixed and processed using the standard methods of fixation by placement in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) and after treating with Triton wetting agent, aspirated for 5 min. and left overnight. The samples were then processed by procedures described by Castillo et al. (2005). The dehydration process was done slowly to discourage hyphal shriveling. The samples were then critical-point dried, gold coated with a sputter coater and images were recorded with a FEI XL 30- ESEM FEG scanning electron microscope.

DNA extraction and sequencing

Total genomic DNA was extracted using a modified cetyl-trimethylammonium bromide (CTAB)-NaCl protocol (Kieser et al., 2000). For each isolate, a loopful of mycelium and spores was scraped from colonies grown on SCA and resuspended in TE buffer as previously described (Conn & Franco, 2004). The DNA was semi-quantified on a 2% agarose gel in 0.5 × Tris-borate-EDTA and visualized by staining with ethidium bromide. PCR was done with the universal 16S primers designed to amplify the region between positions 27 and 765 of the rRNA gene sequence in actinobacteria (Coombs & Franco, 2003). The primers were designated 27f (5’-AGAGTTTGATCMTGGCTCAG-3’) and 765r (5’-CTTGTGATCCTACGGGCTAT-3’) (First Base Lab, Selangor, Malaysia). The PCR was carried out in 50 μL reaction volume with the following reagents: 1.3 μL of 27f (400 ng μL\(^{-1}\)), 1.27 μL of 765f (400 ng μL\(^{-1}\)), 5 μL of PCR buffer, 4 μL of MgCl\(_2\) (25 mM), 2 μL of dNTP mix (40 mM), 34.23 μL of water, 0.2 μL of Taq DNA polymerase (1 U μL\(^{-1}\)) and 2 μL of Template DNA. The reaction mixture was subjected to the following temperature cycling profile: 94 °C for 8 min; followed by 30 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min and finally 72 °C for
10 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc., CA, USA). Then the products were subjected to automated sequencing with the BigDye Terminator Cycle Sequencing Kit from ABI (Applied Biosystem, CA, USA). The temperature cycling profile was identical to the amplification of the region from positions 27 to 765 of the rRNA gene sequence described above. The sequence data from each of the streptomycetes in this study were deposited in GenBank and also compared with other streptomycete gene sequences in GenBank using the BLAST software (BLASTN) at the National Center of Biotechnolgy Information (NCBI) web site (http://wwwncbinihgov/).

Bioassays of the streptomycetes

Each streptomycete isolate was grown as a c. 2 cm colony for 10–14 days on Petri plates containing SCA. Then, small (3 × 3 × 3 mm) plugs of agar containing freshly grown cultures of: Fusarium solani, Aspergillus fumigatus, Mycosphaerella fijiensis, Pythium ultimum, Sclerotinia sclerotiorum, R. solani and Phytophthora erythroseptica were placed about 1.0 cm from the edge of streptomycete colony (Castillo et al., 2007). Yeasts and bacteria, on the other hand, were streaked about 1–1.5 cm from the edge of the colony being tested and these included Saccharomyces cerevisiae, Candida albicans, Bacillus subtilis and Escherichia coli. Most of these organisms were selected because they represent a wide range of plant pathogens as well as certain fungal pathogens of man as well as a representative Gram-positive and Gram-negative bacterium. Growth of the test organisms was evaluated after 24, 48 and 72 h, and recorded as growth, inhibition and no growth as compared with a control plate containing no streptomycete colonies. Finally, a sample of each test organism was taken from the treatment plate and placed on a plate potato dextrose agar (PDA) to determine if the test organism had been killed after having been exposed to it for 72 h. The test was repeated at least thrice.

Fermentation, extraction and assay procedures

Several small blocks of SCA containing the streptomycete colonies were inoculated into 75 mL of potato dextrose (PD) broth for 3 days and eventually transferred onto 600 mL nutrient broth in a 2 L Erlenmeyer flask and incubated for 3 weeks with shaking at 24 °C. Individual culture filtrates were each extracted with three half-volumes of either methylene chloride, n-butanol or ethyl acetate. Each organic solvent extract was then pooled and taken to dryness under flash evaporation at 40 °C. The yield of dry material per liter was varied depending upon which solvent was used but with methylene chloride it was 145 mg (HBB SUK 01), 110 mg (SSP SUK 02), 100 mg (HBPT SUK 03). Known amounts of the dried extract taken up in methanol were then placed directly onto a spot on a PDA plate, dried in the hood, and then the test organism was placed 1.0 cm away from the test spot and eventually evaluated for growth by measuring hyphal development in contrast to an uninoculated control plate. The data are expressed as % of linear growth as compared to the control and repeated thrice.

Results and discussion

Endophytes from Malay plants

Fungi have been previously recovered from some Malay Peninsula associated ethnobotanical plants, however, endophytic streptomycetes have never been reported (Radu & Kqueen, 2002). To this end, surface sterilized tissues from nearly 200 individual plant species were subjected to selective techniques that would result in bacterial growth. Three filamentous bacteria appeared from the following plants: T. grandiflora (family – Aristolochiaceae); Polyalthia spp. (family – Annonaceae), and Mapania sp. (family – Cyperaceae). Each possessed hyphae of a small diameter, i.e. 0.5–0.8 μm, and each was Gram positive. All isolates produced an earthy odor after the formation of an aerial mycelium. Further, SEM data yielded some information about spore formation or potential spore formation of each as being through hyphal fragmentation to produce barrel-shaped spores with a diameter equaling that of the mycelium itself (Fig. 1a–f). Molecular biological analyses of rRNA gene sequence indicated that each isolate was unique among all other Streptomyces spp. appearing in GenBank and the genetic relationship to other organism is presented (Table 1).

Each isolate possessed some biological activity against the fungal test organisms and none was lethal to either the Gram-positive or the Gram-negative bacteria (Table 2). Most importantly, each of these streptomycetes was lethal against some of the most plant pathogenic filamentous fungi that plague the world’s crop plants such as M. fijiensis, Pythium ultimum, S. sclerotiorum, R. solani, and Phytophthora erythroseptica. At least two inhibited Aspergillus fumigatus, a pathogen of man (Table 2). The rRNA gene sequence information of each organism has been submitted to GenBank and accession numbers assigned, and culture deposit numbers given (Table 1). The three organisms were each found near the east coast at Bukit Bauk, Trengganu. Each is listed below and information is given on its observed characteristics (Fig. 1).

MSU 2338- HBB SUK 01: This microbe was recovered from the stems of T. grandiflora. It is a medicinal shrub with antidysestentary properties. This bacterium produced a visible mycelium can be observed after 2 days of incubation on SCA and formation of aerial mycelium after 4 days incubation. The substrate mycelium shows a dry-like opalescence with the production of aerial mycelia after 7 days. The aerial
mycelium was gray and the colony took on a greenish coloration. The SEMs of this organism showed hyphae (0.4–0.5 μm) some of which are intertwining and there is some evidence of an extracellular matrix (Fig. 1a). Some spore formation is occurring via segmentation of the hyphae into barrel-like spores (Fig. 1b). The organism has the greatest similarity, in GenBank, to *Streptomyces fulvoviolaceus* with 94% base sequence similarity (Table 1). In the bioassay test, this organism inhibited *B. subtillus* and killed all plant pathogens tested except *F. solani* (Table 2).

MSU 2337-HTBP-SUK 03: This microbe was recovered from the roots of *Polyalthia* sp., a medicinal tree with reported uses mainly to treat skin infections, high blood pressure and diabetes. The mycelium can be observed 2 days after inoculation on to an SCA plate and after 4 days aerial mycelia began to appear. The aerial mycelium is whitish and eventually the colony develops a dark brown coloration. All isolates produce an earthy odor after aerial mycelium formed. The SEMs of this organism showed hyphae (0.5–0.6 μm) some of which are intertwining and there is evidence of an extracellular matrix (Fig. 1c). Spore formation, into barrel-like spores, is occurring via segmentation of the hyphae (Fig. 1d). The organism has the greatest similarity, in GenBank, to *Streptomyces coelicolor* with 96% base sequence similarity (Table 1). In the bioassay test, this organism inhibited *Aspergillus fumigatus* and killed all plant pathogens tested except *F. solani* (Table 2).

MSU 2339- SSP SUK 02: This endophyte was recovered from the stems of *Mapania* spp., known as spikesedge and apparently has antiprotozoal activity. The SSP SUK02

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**Table 1.** Identification of the Mayalsian endophytic *Streptomyces* sp. isolates based on partial sequencing of the 750 bp region of the rRNA gene sequence along with deposit numbers and the organism (Streptomycete) most closely related

<table>
<thead>
<tr>
<th>Malaysian endophytic streptomycete</th>
<th>Deposit number in GenBank</th>
<th>Closet related streptomycete</th>
<th>% Similarity to rRNA gene sequence</th>
<th>Score (bits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBB SUK 01 MSU 2338</td>
<td>EF383121</td>
<td><em>Streptomyces fulvoviolaceus</em></td>
<td>94</td>
<td>655</td>
</tr>
<tr>
<td>SSP SUK 02 MSU 2339</td>
<td>EF411192</td>
<td><em>Streptomyces caelestis</em></td>
<td>93</td>
<td>648</td>
</tr>
<tr>
<td>HTBP SUK 03 MSU 2337</td>
<td>EF411193</td>
<td><em>Streptomyces coelicolor</em></td>
<td>96</td>
<td>669</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Scanning electron microscopy of streptomycetes from the Malay Peninsula. Hyphae (a) and spores (b) of MSU 2338-HBB-SUK 01, hyphae (c) and spores (d) of MSU 2337-HTBP-SUK 03, and hyphae (e) and spores (f) of MSU 2339-SPP-SUK 02 are shown.
Bacillus subtilis
Petri plate containing PDA to determine its viability. There was no killing of
was not recovered. The viability test was done by placing the original inoculum plug, or streaking representative samples of the yeasts or bacteria, ona
the growth of the test organism was evaluated. The indication ‘dead’ means no growth of the test organism during the test period and the organism
Each streptomycete was grown for 7–10 days on nutrient agar before being challenged with the test organism. During the course of 3 days of exposure,
shows formation of substrate mycelium after 3 days of incubation on SCA and at 7 days, aerial mycelium can be observed on SCA. The color of aerial mycelium is white and the colony was chalky. Towards the center of the colony, abundant spore formation occurred via hyphal segmentation with the spore diameter being c. 0.4 × 1.1 μm (Fig. 1e and f). This organism killed several major plant pathogens and was inhibitory to B. subtilis (Table 2). Genetically, its most closely related streptomycete is Streptomyces caelestis at 93% similarity (Table 1).

**Biological activity in culture extracts**

Each streptomycete was grown in culture and then the filtered culture fluid extracted with one of three solvents. The dried organic solvent extract was then subjected to a bioassay test to determine if any biological activity could be successfully extracted from the culture fluid. In each case, some biological activity was extractable with each organic solvent, but the one extracting the greatest amount of material with the greatest activity was methylene chloride. As an example, when 3.0 mg of the extractable solids of the streptomycete HTBP SUK 03, representing each organic solvent, was subjected to the PDA plate assay; the methylene chloride extract showed the greatest biological activity with the ethyl acetate extract closely behind (Table 3). The extracts of the other streptomycetes showed a similar profile (not shown). Overall, it appears that the bioactive component(s) have mostly a lipophilic profile given their organic solvent preference (Table 3). The extracts were then subjected to further purification by silica gel column chromatography and it was possible to acquire bioactivity from the

**Table 2. Biological activities of the Malaysian endophytic Streptomyces**

<table>
<thead>
<tr>
<th>Test organism</th>
<th>HBB SUK 01 (from Thottea grandiflora)</th>
<th>SSP SUK 02 (from Mapania spp)</th>
<th>HTBP SUK 03 (from Polyalthia spp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>No effect</td>
<td>75–83%</td>
<td>28–35%</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Inhibited</td>
<td>Inhibited</td>
<td>No effect</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>60–66%</td>
<td>50–58%</td>
<td>40–50%</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>50–66%</td>
<td>Dead</td>
<td>50–60%</td>
</tr>
<tr>
<td>Mycosphaerella fijiensis</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>Pythium ultimum</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>Phytophthora erythroseptica</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
</tbody>
</table>

Each streptomycete was grown for 7–10 days on nutrient agar before being challenged with the test organism. During the course of 3 days of exposure, the growth of the test organism was evaluated. The indication ‘dead’ means no growth of the test organism during the test period and the organism was not recovered. The viability test was done by placing the original inoculum plug, or streaking representative samples of the yeasts or bacteria, on a Petri plate containing PDA to determine its viability. There was no killing of Bacillus subtilis, and no effect was noticed on Candida albicans and Escherichia coli for all isolates. The numbers given represents the percentage (in range) inhibition of measurable growth over the control in three separate tests and the test organism, in each of these cases, remained alive ‘Inhibited’ indicates that the culture did not grow as fast or as well as the control and because of the nature of the culture, no linear measurement could be made.

**Table 3. Percentage of inhibition of the some test fungi using the various organic solvent extracts of HTBP SUK 03 at a concentration of 3 mg 60 –1 μL applied to a PDA plate and dried**

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Ethyl acetate (%)</th>
<th>Methylene chloride (%)</th>
<th>n-Butanol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pythium ultimum</td>
<td>80–90</td>
<td>80–90</td>
<td>10–20</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>75–85</td>
<td>60–75</td>
<td>75–81</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>60–75</td>
<td>70–85</td>
<td>10–20</td>
</tr>
<tr>
<td>Phytophthora erythroseptica</td>
<td>85–95</td>
<td>85–95</td>
<td>45–56</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>40–50</td>
<td>45–55</td>
<td>15–22</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>65–85</td>
<td>85–95</td>
<td>15–20</td>
</tr>
</tbody>
</table>

The growth is reported as % of control. The material represented the extract each from ethyl acetate, methylene chloride and n-butanol with the various plant/human pathogenic fungi as test organisms.

**Acknowledgements**

The authors appreciate the help and assistance of the UNESCO fellowship fund and The University of

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Kebangsaan, Malaysia for providing support to Nora Zin to have a work leave to Montana State University. Financial support was also supplied by the Montana Department of Commerce-Board of Research and Commercialization Technology and the Dole Fruit Company. The Montana Agricultural Experiment Station also provided financial support.

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