

EXPLORATION OF MALAYSIAN SOIL ACTINOBACTERIA FOR NEW ANTIBIOTICS USING INTEGRATED APPROACH

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INTRODUCTION

Secondary metabolites produced by the gram positive actinobacteria are highly potent and biologically active and remain a powerful source for pharmaceutical discovery (Umashankar *et al.*, 2010). Studies have shown that dipterocarp forests in Southeast Asian countries have high actinobacteria diversity (Wang *et al.*, 1999). However, with few reports addressing the diversity of actinobacteria in tropical Malaysian forest soil, the vast possibilities of using indigenous microbes as sources for new bioactive compounds and other applied research have not been adequately tapped. With the declining rate of discovery of new antibiotics from the genus *Streptomyces*, increased emphasis is placed on targeting the rare actinobacteria group that produce commercially valuable antibiotics (Boudjella *et al.*, 2006). In this study, members of the family *Streptosporangiaceae* are targeted. The use of specific probes as selective PCR amplification primers offers an alternative approach for the rapid identification of large numbers of strains belonging to specific genera under this family (Monciardini *et al.*, 2002). This study is a collaborative effort between the Forest Research Institute Malaysia (FRIM) and Nimura Genetic Solutions (NGS) to use various selective isolation techniques to explore the diversity of actinobacteria isolated from natural forest areas, re-planted forest areas and tin tailings at FRIM campus and its forest substations. The second objective is to establish the FRIM Actinobacteria Culture Collection (FACC) and database for future R&D leading to drug discovery. In order to achieve the second objective, a multi-dimensional approach based on biochemical typing, gene typing and chemical profiling using HPLC-DAD analysis were used to target unexploited genera from the FACC collection to search for potential producers of antimicrobial compounds.

MATERIALS AND METHODS

A total of 273 soil samples were collected from FRIM main campus (Bukit Lagong area), and FRIM substations (Pasoh, Bidor, Mata Ayer and Segamat). A GPS system was used to identify soil collection sites. In-house isolation methods were used where samples were chemically or physically **pre-treated and** serially diluted using sterile saline. Soil suspensions were spread on a number of selective agar media (Hayakawa, 2008). Actinobacteria colonies forming on the isolation plates were isolated and characterised based on macromorphological characteristics into the following genera/groups: *Streptomyces*, *Micromonospora*, *Actinoplanes*, *Nocardioform* and oligosporic-type. Biochemical typing (BCT) was done by scoring the growth response of the isolates in the presence of selected amino acids and antibiotics compared to growth on control plates with basal media. Spore suspensions of isolates in 20% (w/v) glycerol were stored in cryovials at -80°C. Colony direct PCR using intact cells (Ishikawa *et al.*, 2000) with

genus specific primers was carried out on a total of 336 isolates characterised as oligosporic-type using standard PCR conditions. Validated genus-specific primers NOM F1 and NOM R2 used for rapid identification of *Nonomuraea* spp. and closely related genera were derived from 16S rRNA gene sequence. Controls without bacterial DNA and DNA isolated from non-target organisms were included in each PCR experiments. Unique or uncommon isolates were selected from the *Nonomuraea* group identified by the PCR studies by comparing their BCT profiles with those of reference strains from the *Streptosporangiaceae* family. Submerged cultures and crude extracts of the selected isolates were prepared as described by Getha *et al.* (2009). Crude extracts were evaluated for antimicrobial activity against a panel of test microbes using paper-disc assay method. Chemical profiling of extracts showing strong antimicrobial activity was done using HPLC equipped with a diode-array detector (Agilent 1100 Series; reversed phase analytical column ZORBAX XDB-C18; Agilent Technologies, USA). Multiple wavelength monitoring was performed and UV-visible spectra measured at 200, 230, 254 and 320 nm. The UV-visible spectra of chromatographic peaks were compared with those of reference compounds, mostly antibiotics, from in-house HPLC-UV-vis database (Fiedler, 1993).

RESULTS AND DISCUSSION

A total of 2286 isolates of actinobacteria were isolated using 10 different isolation methods which include either the chemical or physical pretreatment techniques in combination with enrichment techniques that use different agar media with selective antimicrobial agents. Isolates presumptively assigned to the *Streptomyces*-like group were distinguished from other bacterial colonies growing on the isolation plates by their characteristic colony and pigmentation properties. Isolates classified in the *Micromonospora* and *Actinoplanes* group formed small, compact and waxy or slimy colonies, and produce substrate mycelium but lacked aerial mycelium. Their substrate mycelium is orange to orange-brown and, in *Micromonospora*, spores when present blackened the surface of the colonies. Isolates with the oligosporic-type morphology identified by distinctive macromorphological characteristics, comprised of genera from the family *Streptosporangiaceae*. The majority of colonies developed on starch-casein (SC) agar are *Streptomyces* spp. The fast growing streptomycetes retarded the slower growing actinobacteria such as *Micromonospora* and other genera, from growing out on the SC agar plates. The presumptive rare actinobacteria, including slow growers such as *Micromonospora*, *Actinoplanes* and *Streptosporangium*, grew well on the humic acid-vitamins (HV) agar. Hayakawa and Nonomura (1987) have demonstrated that HV agar was useful for efficient recovery and adequate growth of streptomycetes and various rare actinobacteria, while restricting growth of non-filamentous bacteria. The HV agar also supported good sporulation for these actinobacteria, which offers a considerable advantage for rapid morphological identification. Selective agents like gentamycin, tunicamycin and novobiocin have been described for the isolation of specific genera of soil actinobacteria (Wang *et al.*, 1999). Therefore, selective antibiotics were used to enhance the isolation of rare actinobacteria in this study. Besides isolation media and incubation conditions, the selectivity of different groups of actinobacteria was also influenced by the nature of sample pretreatment regimes. Physical pretreatment by heating air-dried soil samples reduced the numbers of filamentous bacteria and streptomycetes on isolation plates. The chemical pretreatment method resulted in a higher number of isolates obtained compared to physical pretreatment. The chemical method was also effective in isolating a higher diversity of actinobacteria genera.

In all studied areas, the predominant genus was *Streptomyces*, occurring at almost 50% or more of the total isolated. Isolates grouped as oligosporic-type were most frequently isolated from lowland dipterocarp forest areas in Pasoh and Mata Ayer. These isolates have distinctive

morphological characteristics and are comprised of at least nine genera from the family *Streptosporangiaceae*. In the present study, isolates belonging to *Nonomuraea* and closely related genera are targeted since they have been reported as potential producers of useful antibiotics. Further, the genus *Nonomuraea* has high isolation ratio in Malaysian soil and is yet to be fully exploited for the discovery of novel compounds (Muramatsu *et al.*, 2003). A total of 255 isolates were identified based on PCR-amplification using genus-specific primers as members of the genus *Nonomuraea* and closely related genus. By comparing the biochemical typing (BCT) profiles, isolates selected as unique/uncommon strains for fermentation showed that a higher percentage of these strains showed bioactivity against gram positive bacteria, compared to antifungal activity. Chemical profiling of the active crude extracts using HPLC-DAD analysis showed that by comparing UV-visible absorbance spectra and retention time of HPLC peaks with those of antibiotics and other metabolites from in-house compound library, a number of known compounds were shown to be present in some of the active extracts. Interestingly, the HPLC-DAD elution profiles of eight active extracts gave major peaks that did not match any data in the spectral libraries. These interesting extracts will be studied further to isolate and identify the active compounds, and to check on their novelty.

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