POLISH POLAR RESEARCH
vol. 36, no. 1, pp. 67–78, 2015 doi: 10.1515/popore-2015-0001

Isolation, identification and screening of Actinobacteria in volcanic soil of Deception Island (the Antarctic) for antimicrobial metabolites

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Abstract: This project aimed to isolate and characterize volcanic soil Actinobacteria from Deception Island, Antarctic. A total of twenty-four Actinobacteria strains were isolated using four different isolation media (Starch casein agar, R2 agar, Actinomycete isolation agar, Streptomyces agar) and characterized basing on 16S rRNA gene sequences. Tests for secondary metabolites were performed using well diffusion method to detect antimicrobial activities against eight different pathogens, namely *Staphyloccocus aureus* ATCC 33591, *Bacillus megaterium*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *S. enterica* serotype Enteritidis, *S. enterica* serotype Paratyphi ATCC 9150, *S. enterica* serotype Typhimurium ATCC 14028 and *Vibrio cholerae*. Antimicrobial properties were detected against *Salmonella paratyphi* A and *Salmonella typhimurium* at the concentration of 0.3092±0.08 g/ml. The bioactive strains were identified as *Gordonia terrae*, *Leifsonia soli* and *Terrabacter lapilli.* Results from this study showed that the soil of Deception Island is likely a good source of isolation for Actinobacteria. The volcanic soil Actinobacteria are potentially rich source for discovery of antimicrobial compounds.

Key words: Antarctic, Actinobacteria, secondary metabolites, 16S, diffusion assay, selective isolation media.

Introduction

Deception Island is a ring-shaped caldera-type volcanic island located at the south-western end of the South Shetland Islands and north-west of the Antarctic Peninsula. It is currently classified as being restless with a significant risk, but no

eruption is anticipated as the volcano is in its quiet, dormant state (Deception Island Management Group 2005).

Actinobacteria is a class of Gram-positive bacteria with high guanine and cytosine (G+C) content. Their morphology is highly pleomorphic; growing as filaments that branch into radiate or star-like shape, or form irregularly-shaped rods or cocci. They are primarily saprophytic and are known to contribute in nutrient turnover, using many available nutrient sources for their development (Solano *et al*. 2009).

Actinobacteria are widespread in soil and are able to produce various useful secondary metabolites and compounds containing various properties (Wellington *et al*. 1992; Poornima and Ponmurugan 2006). Approximately 75% of commercially useful antibiotics are derived from the genus *Streptomyces*. However, in recent years, the isolation of well-known Actinobacteria, such as *Streptomyces* from various different environments, was found to be producing similar compounds. This has resulted in vital demand for new leading structures in pharmacology, which in turn has made the exploration of new habitats in unusual environments essential to discover novel Actinobacteria and metabolites (Barakate *et al*. 2002; Saadoun and Gharaibeh 2003; Newman and Cragg 2007; Thakur *et al*. 2007). These strategies are likely to retrieve microorganisms that have the capacity to produce useful metabolites (Lazzarini *et al*. 2000; Srivibool and Sukchotiratana 2006; Thakur *et al*. 2007; Okoro *et al*. 2009).

Materials and methods

Environmental Sampling. — The volcanic soil sample was collected from 62°58'42.2" S 60°42'71.5" W with pH 5 and temperature of 94°C at the time of collection on 26 January 2008 during the Malaysian Antarctic Expedition. Soils were sampled into sterile container using an aseptic metal trowel, and kept in the dark for transport to Malaysia. Soils were subsequently stored at -20°C.

Selective isolation of Actinobacteria from environmental samples. — Total of 2 g air dried soil samples were ground with mortar and pestle. The soil samples were diluted with ultrapure water to achieve 10^{-1} dilution. The mixture was subjected to sonication for 30 seconds to release microorganisms bound to the soil particles (Labeda and Shearer 1990) and shaken at room temperature for one hour. Subsequently, serial dilution up to 10^{-2} and 10^{-3} were prepared. The four isolation media – starch casein agar (SCA) (Merck, Germany), R2A agar (Merck, Germany); actinomycete isolation agar (AIA) (BD Difco™, USA) and streptomyces agar (SA) (HiMedia, Mumbai) – were prepared and supplemented with cycloheximide (0.05 g/l) except for R2A to inhibit the growth of fungi.

Plate count was carried out using the spread plate technique. Aliquots of one hundred µl of the 10^{-1}, 10^{-2}, 10^{-3} suspensions were spread onto isolation media and incubated at 5°C, 26°C and 45°C. The growth on the agars was observed and re-

corded for thirty days. At 5°C, psychrophilic bacteria were aimed to be isolated. This was to emulate the cold temperature of the South Shetlands. 26°C is the temperature usually applied to isolate actinobacteria because majority of described actinobacteria are mesophiles with optimum growth temperatures in, or near, this temperature (Labeda and Shearer 1990). As the temperature of the soil at the time of collection was 95°C, the plates were incubated at 45°C to isolate thermophiles. This lower temperature compared to the original temperature was to prevent the agar from drying too fast. Special attention should be paid to the plates incubated at this temperature as the bacteria tend to reach stationary growth phase very rapidly and then undergo autolysis (Labeda and Shearer 1990). This phenomenon could be observed when some of the colonies seemed to "disappear" after a few days of observation.

Colonies putatively identified as Actinobacteria were picked and kept in their respective semi-solid agars supplemented with cycloheximide (0.05 g/l). They were distinguished based on the morphology, usually their colours, and in some cases, the ability to form inhibition zones. PRIMER6 software utilizing Bray-Curtis non-metric multi-dimensional scaling similarity analysis was applied to investigate the distribution and similarity of Actinobacteria isolated on different media.

Preparation of crude extracts. — A total of twenty-four actinobacteria-like strains were grown in actinomyces broth (BD BBL™, USA) supplemented with cycloheximide (0.05 g/l), nalidixic acid (0.02 g/l) and nystatin (0.05 g/l). These cultures were grown in a rotary shaker at 200 rpm for seven days. The resulting culture broths (approximately 50 ml), obtained following growth of each isolate in the culture media, separated from the cell by centrifugation at 10.000 g for 15 min. The cell-free supernatant was collected and freeze-dried. Freeze-dried materials were resuspended into appropriate concentration for antimicrobial activity by agar well diffusion method against test microorganisms (Grammer 1976).

The test organisms were two Gram-positive (*Staphyloccocus aureus* ATCC 33591 and *Bacillus megaterium*) and six Gram-negative (*Enterobacter cloacae*, *Klebsiella oxytoca*, *Salmonella enterica*, serotypes: Enteritidis, Paratyphi and Typhimurium ATCC 14028 and *Vibrio cholerae*) bacteria, respectively.

Well diffusion assays for antimicrobial activity. — By using a sterile cork borer, wells were punctured in appropriate agar medium plates previously seeded with one of the test organisms. A total of eighty microliter of supernatant of each isolate was administrated in each well. Plates were kept at 4°C for 2 hours to allow the diffusion of produced antimicrobial metabolites before incubating at 37°C for 24 hours. The diameter of the zone of inhibition was then recorded on day-three. A clear zone suggests that the sample has properties that can inhibit the growth of the test organism. No clear zone indicates complete resistance.

The use of agar in radial diffusion method in antimicrobial screening is more advantageous when compared to the micro-gel well diffusion assay and microtiter

broth dilution method in that microbial heterogeneity or contamination can be detected readily by observing the nature of bacterial growth, as opposed to a broth where a contamination would not be directly discovered (Du Toit and Rautenbach 2000).

Genomic DNA extractions and PCR amplification. — Genomic DNA was extracted using the conventional phenol:chloroform method. 3ml of positive broth was harvested by centrifuging for 5 minutes at 12.000 g. The pellet was resuspended in 500 µl of TSE buffer, and incubated at 37°C for 30 minutes. 300 µl of 2% SDS was added and vortexed for 20 seconds. After adding 500 µl of phenol:chloroform (25:24), the tube was again vortexed and centrifuged at 12.000 g for 10 minutes at 4°C. This resulted in an upper aqueous layer containing the DNA and an interface above the organic layer that contains the precipitated proteins. Repeated extraction was carried out until no such interface observed (Santella 2006). The aqueous phase (supernatant) was then transferred to a new tube and equal volume of chloroform was added. After vortexing and centrifuging at 12.000g for 5 minutes, the aqueous phase was transferred to another new tube. 0.1 volume of 3M sodium acetate was added. 1 volume of isopropanol was added after vortexing. Next, the tube was centrifuged at 12.000 g for 5 minutes at 4°C. The supernatant was gently discarded. 500 µl of 75% ethanol was added. The supernatant was again discarded after centrifugation at 12.000 g for 5 minutes. The pellet was air-dried and resuspended with 5 0µl ultrapure water. The DNA yield and quality was assessed by 0.8% (w/v) agarose gel electrophoresis following by DNA quantification using a Biophotometer (Eppendorf, Germany) and ratio A_{260}/A_{280} was measured.

Touchdown PCR with actinobacteria-specific primer was carried out to confirm whether the isolates were actinobacteria. PCR reaction (20 µl) contained the following: 12.7 µl of sterile distilled water, 2.0 µl of 10X buffer (350 mM Tris-HCl pH 9.0, 250 mM KCl, 35 mM $MgCl_2$, enhancer solution, *i-Taq™* Plus PCR Buffer, iNtRON Biotechnology, Korea) containing 2.0 µl of dNTPs (2.5 mM/each, iNtRON Biotechnology, Korea), 1.0 µl of forward primer (5'-CGC GGC CTA TCA GCT TGT TG-3'), 1.0 µl reverse primer (5'-CCG TAC TCC CCA GGC GGG G-3'), 0.3 µl of Taq polymerase (5U/µl, *i-Taq™* Plus DNA Polymerase, iNtRON Biotechnology, Korea) and 1.0 µl of template DNA. The reactions were performed on a Palm cycler (Corbett Research, Australia) with the following cycling program: 95°C for 4 minutes, followed by one amplification cycle of 95°C for 45 seconds, 72°C for 45 seconds and 72°C for 1 minute. After that, the touchdown process was carried out for eight cycles by decreasing the annealing temperature for 0.5°C per cycle from 72°C to 68°C. The PCR was then proceeded with another 26 rounds of the initial amplification cycle. The reaction was ended by final extension at 72°C for 5 minutes. The product size was approximately 640 bp.

The 16S rDNA amplification was applied for the molecular identification. The forward primer used was p27f (5'-AGA GTT TGA TCC TGG CTC AG-3') whereas the reverse primer was p1492r (5'-TAC GGC TAC CTT GTT ACG ACT

T-3'). PCR reaction (20 µl) contained the following: 12.8 µl sterile distilled water, 2.0 µl 10X buffer (350 mM Tris-HCl pH 9.0, 250 mM KCl, 35mM $MgCl_2$, enhancer solution, *i-Taq™* Plus PCR Buffer, iNtRON Biotechnology, Korea), 2.0 µl dNTPs (2.5mM/each, iNtRON Biotechnology, Korea), 1.0 µl of each primer, 0.2 µl of Taq polymerase (5U/µl, *i-Taq™* Plus DNA Polymerase, iNtRON Biotechnology, Korea) and 1.0 µl template DNA. The reactions were performed on a Palm cycler (Corbett Research, Australia) with the following cycling program: 95°C for 4 minutes followed by 35 cycles of 95°C for 45 seconds, 68.5°C for 45 seconds and 72°C for 1 minute, and 8 minutes extension at 72°C. The product size was approximately 1380 bp.

A total of 20 µl of the PCR product was resolved on 1.5% agarose gel electrophoresis and stained with ethidium bromide (0.5 µg/ml) prior gel documentation (Alpha Imager, Alpha Innotech, USA) process. The desired banding size on agarose gel were purified using GeneJET™ Gel Extraction Kit (Fermentas, Ontario, Canada) according to the manufacturer's protocol and subjected to sequencing using an ABI PRISM® 3100 DNA sequencer (Applied Biosystems). The calculations of level of sequence similarity for the 16S rRNA gene sequences were carried out using the EzTaxon server 2.1 (Chun *et al*. 2007).

Results

All agar plates showed growth except for Streptomyces agar of dilution 10^{-3} at the temperature of 45°C. The colour of the colonies were mostly in the multi-membered colour groups of white and yellow, with some being red, orange and transparent. There were also colonies in the single-membered colour groups of brown and blue. The sizes ranged from pinpoint to 5 mm, some reaching 17 mm and even 85 mm. They were usually round and smooth or chalky, and may be pigmented. Several showed inhibition halos. Colonies with radial-like shape and irregular margin could also be observed.

A description of the cluster diagrams from Bray-Curtis non-metric multi-dimensional similarity analysis between the four media used is in the Table 1. An example of the cluster diagram with its legend is illustrated in Fig. 1. Bray-Curtis Similarity Analysis is commonly used in community ecology. It is utilized here to compare and illustrate the percentage of similar colonies among the four media ranging from twenty to eighty percent. Generally, the bacteria grow differently on each agar at different temperatures and dilution factors. However, there are trends that can be observed. SCA shows the most frequent 40% similarity with either AIA or R2A, while AIA shows the most frequent 20% similarity with either R2A or SA. In six out of the nine incubation conditions, SA shows no similarity at the lowest 20% level with any other agar, indicating that the bacteria that grow on SA may be

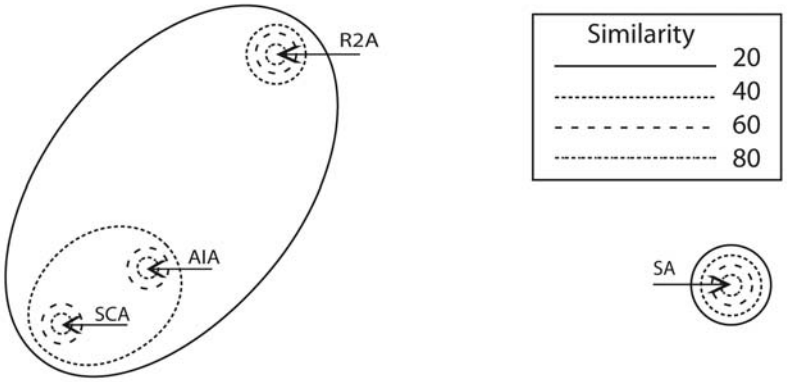

Fig. 1. An example of cluster diagram illustrating the similarity of the colonies grown on the four media with dilution 10^{-1} at 5°C.

unique to SA, or that the bacteria may be present on other agars, but display distinct characteristics when grown on SA.

Nineteen out of 24 isolates that showed positive growth in actinomyces broth, only 14 showed bands when subjected to touchdown PCR with Actinobacteria-specific primer and only 12 out of 14 gave a PCR amplification product of the expected size (~640 bp). However, all the secondary metabolite of the 14

Table 1

Description of the cluster diagrams generated using PRIMER6 Software utilizing Bray-Curtis non-metric multi-dimensional similarity analysis.

Incubation condition	Cluster similarity diagram description
Dilution 10^{-1} at 5°C	At 20%, SA showed no similarity with SCA, AIA and R2A, but SCA, AIA and R2A showed similarity with each other. At 40%, SCA and AIA showed similarity with each other.
Dilution 10^{-1} at 26°C	At 20%, SCA and AIA showed no similarity with each other and with other media, but there was similarity between SA and R2A.
Dilution 10^{-1} at 45°C	At 20%, all four media showed no similarity with each other.
Dilution 10^{-2} at 5°C	At 20%, SCA and SA showed no similarity with each other and with other media, but there was similarity between R2A and AIA.
Dilution 10^{-2} at 26°C	At 20%, AIA showed similarity with SA. At 40%, SCA showed similarity with R2A.
Dilution 10^{-2} at 45°C	At 20%, SA showed no similarity with other media. At 40%, SCA, R2A and AIA showed similarity with each other. At 60%, SCA showed similarity with AIA.
Dilution 10^{-3} at 5°C	At 20%, AIA showed similarity with SA. At 40%, SCA showed similarity with R2A.
Dilution 10^{-3} at 26°C	At 20%, SA showed no similarity with other media, but SCA, R2A and AIA showed similarity with each other.
Dilution 10^{-3} at 45°C	At 20%, SA and R2A showed no similarity with each other and with other media. At 80%, SCA showed similarity with AIA.

SCA, Starch Casein Agar; AIA, Actinomycete Isolation Agar; SA, Streptomyces Agar

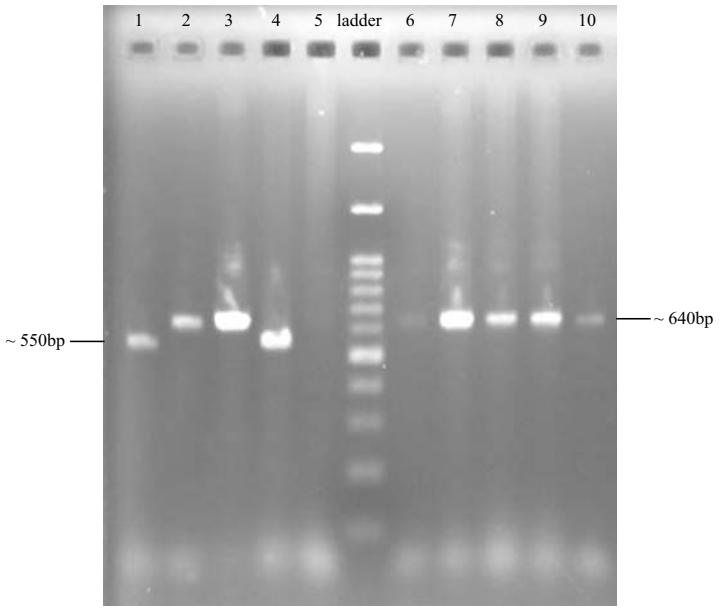

Fig. 2. A representative gel picture obtained using Touchdown PCR for 10 samples. Lanes 1 and 4 showed bands at 550 bp while the others showed bands at 640 bp. The DNA ladder used was 100 bp.

bacteria were proceeded for the antibacterial assay. A representative gel picture is shown in Fig. 2.

There was a total of 28 samples of freeze-dried secondary metabolite from the 14 isolates as the secondary metabolite that were collected at two time points. At the initial concentration of 0.025 g/ml, no inhibition zone was observed. When the concentration was increased to 0.3092±0.08 g/ml, inhibition against *S. enterica* serotype Paratyphi A and *S. enterica* serotype Enteritidis were observed. Examples of plate pictures depicting inhibition are in Figs 3 and 4. Five isolates (with designated sample number 8, 10, 13, 18 and 19) that showed significant inhibition were amplified using p27f and p1492r primers prior to gel extraction and 16S rDNA sequencing. After comparing to the database on EZ Taxon, sample 8 was found to be *Gordonia terrae* at 99.9% similarity, sample 10 was *Leifsonia soli* at 99.9% similarity, and the remaining three were found to be *Terrabacter lapilli* at 99.2%, 99.1% and 99.1% similarity, respectively.

G. terrae (sample 8) was isolated from actinomycete isolation agar incubated at 26°C with dilution 10^{-2}. The colony was yellow in colour and changed to dark yellow, eventually becoming orange or brown at the size of 3 mm. It was round and Gram-stain showed the picture of rod-shaped bacteria. *L. soli* (sample 10) was isolated from Streptomyces agar incubated at 45°C with dilution 10^{-1}. The colony colour changed from whitish to yellowish and pigmented. The size ranged from 7 to 13 mm. The shape was radial with irregular margin and inhibition halos were present. It was rod-shaped as well. *T. lapilli* (sample 13) was isolated from Streptomyces agar

Fig. 3. Inhibition shown against *Staphyloccocus enterica* serotype Paratyphi A by secondary metabolites labelled as 13b, 18b and 19b (14 mm, 14 mm and 12 mm respectively). Sample 17b showed no inhibition.

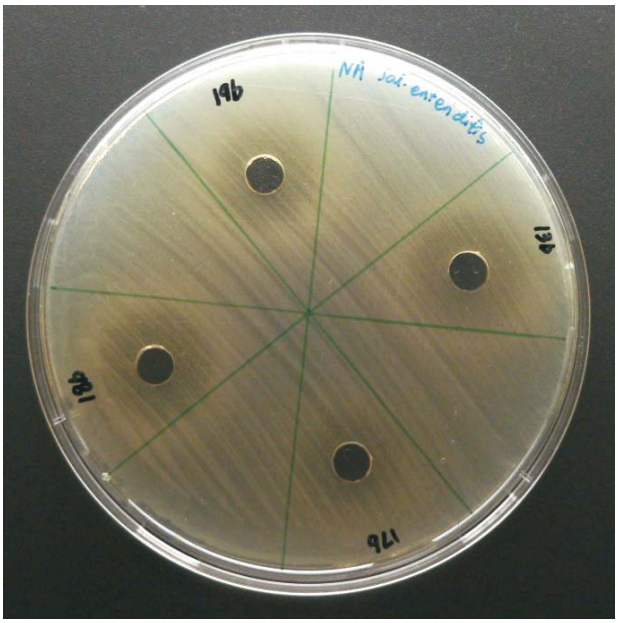

Fig. 4. Inhibition shown against *Staphyloccocus enterica* serotype Enteritidis by secondary metabolites labelled as 13b, 17b, 18b and 19b with the zone diameter of 16 mm, 10 mm, 17 mm and 14 mm respectively.

incubated at 5°C with dilution 10^{-3}. The colonies were round and bright yellow, about 1 to 2 mm. It was rod-shaped. *T. lapilli* (sample 18) was isolated from Streptomyces agar incubated at 5°C with dilution 10^{-3}. The colonies were 1 to 2 mm and white in colour. It was rod in shape, too. *T. lapilli* (sample 19) was isolated from R2A agar incubated at 5°C with dilution 10^{-3}. The colonies were white, round and smooth, and 2 to 4 mm in size. The Gram-stain showed rod-shaped bacteria.

Discussion

Based on the plurality of colour groups observed, Actinobacteria diversity can be predicted (Okoro *et al*. 2009). The more colours that can be observed on the media, the more diverse are the bacteria. Well diffusion assay for screening of antibacterial property is relatively a simple and efficient way of screening for antibacterial property. No inhibition zones were visible at the initial concentration of 0.025 g/ml. This result may indicate complete resistance (Srivibool and Sukchotiratana 2006), or possibly be attributed to the fact that at very low concentration, the secondary metabolite might not diffuse far enough into the gel to form a visible inhibition zone (Du Toit and Rautenbach 2000).

When the concentration was increased to 0.3092±0.08 g/ml, inhibition was seen against *S. enterica* serotype Paratyphi A and *S. enterica* serotype Enteritidis. Normally, a large zone indicates more effective antimicrobial activity or greater diffusibility of the substance, or both. On the other hand, a narrow zone might not indicate that the substance is not potent enough, but that it could not diffuse well into the medium because it was a non-polar substance or composed of rather non-polar components (Srivibool and Sukchotiratana 2006).

The preliminary Antibacterial screening discovered some active strains. The percentage of active strains discovered were low due to the fact that isolation method was not able to cater for the growth of all Actinobacteria at the same incubation time and conditions. Furthermore, various factors were involved in affecting the production of antimicrobial properties from Actinobacteria, such as chemical and biological environment, the sampling size, the probability of right sample to be inoculated, the medium and the methods of screening (Srivibool and Sukchotiratana 2006). Hence, different specific antimicrobial-producing strains of Actinobacteria need different kinds of media for producing substances.

Broadly, *Gordonia* species are Gram-positive coryneform bacteria that are ubiquitous in the environment and often found in soil and water (Grisold *et al*. 2007). They have also been isolated from soil of North Pacific and Caribbean Coasts (Solano *et al*. 2009). *G. terrae* is in the order Actinomycetales, suborder Corynebacterineae. It was first described as the then newly-proposed genus *Gordonia* occurring in sputa of patients with pulmonary disease and in soil. It was slightly acid-fast. On egg media and Sauton agar, the colonies were pinkish or red-

dish (rarely orange) when incubated aerobically and remained light brownish or creamy if the air was limited. It grew at 28°C and 37°C but not at 45°C. Three species, *G. bronchialis*, *G. rubra* and *G. terrae* were described (Tsukamura 1971). Seventeen years later, it was described by Stackebrandt *et al*. (1988) as *Rhodococcus terrae*.

Grisold *et al*. (2007) reported that to date, there has been six case reports of bloodstream infections caused by *G. terrae* found in the literature, and all of the infections occurred in the United States. The isolate grew on blood and chocolate agar, with salmon-to-orange colonies after 48 hours of incubation. Other than this, there are other sites of infection, such as one case of meningitis and brain abscesses in an immunocompetent woman (Drancourt *et al*. 1994) and granulomatous mastitis following nipple piercing (Zardawi *et al*. 2004). It was concluded that eventually *G. terrae* may cause catheter-related bacteraemia, particularly in patients with severe underlying diseases, and proposed that *G. terrae* should be considered a rare but potential pathogen in both immunocompromised and immunocompetent patients.

In spite of these, there is a good point about this strain. The presence of diverse *Gordonia* populations in heavily oil-contaminated soils indicates that the strains are capable of utilizing heavy oil as a source of carbon for their survival and growth. It was the second most dominant strain in all the heavily oil-contaminated soil samples (Shen *et al*. 2008). *G. terrae* was also capable of degrading long-chain *n*-alkanes and *c*-alkanes after enrichment with long-chain hydrocarbons (waste car engine oil, base oil or the *c*-alkane fraction of base oil) as the sole carbon and energy source (Kubota *et al*. 2008).

Most *Gordonia* species were isolated due to their abilities to degrade xenobiotics, environmental pollutants, or otherwise slowly biodegradable natural polymers as well as to transform or synthesize possibly useful compounds. The variety of chemical compounds being transformed, biodegraded and synthesized by Gordoniae makes these bacteria potentially useful for environmental and industrial biotechnology. However, because some Gordoniae are opportunistic pathogens as mentioned above, their application in the environment may be restricted in some cases (Arenskötter *et al*. 2004).

L. soli was isolated from teak rhizosphere soil and described as yellow pigmented, Gram positive, aerobic, motile and short rod-shaped bacteria (Madhaiyan *et al*. 2010). It is in the order Micrococcineae, suborder Microbacteriaceae.

T. lapilli – order Micrococcineae, suborder Intrasporangiaceae – had been isolated from a small stone collected from an agricultural field in Jeju, Republic of Korea. It was described as strictly aerobic, Gram-positive, non-motile short rods. The colonies were bright yellow, circular, smooth and translucent (Lee *et al*. 2008). Apart from this, the genus *Terrabacter* has also been isolated from soil of the Carribean Coast (Solano *et al*. 2009).

Acknowledgements. — This work was supported by Malaysia Antarctic Research Program, Academy of Sciences Malaysia and Universiti Putra Malaysia Research Universiti Grant Scheme (05-01-11-1219RU).

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Received 2 June 2011
Accepted 15 May 2013