MONASH University

DISCOVERY OF NOVEL RARE ACTINOBACTERIA ISOLATED FROM MANGROVE ENVIRONMENTS IN THE EAST COAST OF PENINSULAR MALAYSIA

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Honours Degree of Bachelor of Science (Biotechnology)

A thesis submitted for the degree of Doctor of Philosophy at

Monash University in 2016

Jeffrey Cheah School of Medicine and Health Sciences

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THESIS INCLUDING PUBLISHED WORKS DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes **three** original papers published in peer reviewed journals and **one** manuscript (under review). The core theme of the thesis is discovery of novel bacteria from mangrove sediment of Tanjung Lumpur, Pahang, Malaysia and the antibacterial, neuroprotective and cytotoxic activity of the discovered rare actinobacteria extracts. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Jeffrey Cheah School of Medicine and Health Sciences under the supervision of Dr. Lee Learn Han.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 3-6, my contribution to the work involved the following:

Thesis chapter	Publication title	Status	Nature and extent of candidate's contribution
3	*Microbacterium mangrovi* sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil	Published in International Journal of Systematic and Evolutionary Microbiology	55%. Experimental conduct, laboratory analysis, data collection
4	*Sinomonas humi* sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil	Published in International Journal of Systematic and Evolutionary Microbiology	55%. Experimental conduct, laboratory analysis, data collection
5	*Monashia flava* gen. nov., sp. nov., a novel actinobacterium of the family *Intrasporangiaceae*	Published in International Journal of Systematic and Evolutionary Microbiology	55%. Experimental conduct, laboratory analysis, data collection
6	Antibacterial, anticancer and neuroprotective activities of rare actinobacteria from mangrove forest soils	Indian Journal of Microbiology (Under review) (INJM-D-16-00327)	75%. Experimental conduct, laboratory analysis, data collection and preparation of manuscript

I have / have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signature 

Date: 13 May 2016

ACKNOWLEDGEMENT

~In the Name of Allah, the Most Gracious, the Most Merciful~

I would like to express my sincere gratitude to my main supervisor, Dr. Lee Learn Han for the continuous support along my PhD study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me during my time of research and thesis writing. Besides my advisor, I would like to thank to my co-supervisors; Professor Dr. Iekhsan Othman, Associate Professor Dr. Fang Chee Mun, and Associate Professor Dr. Chan Kok Gan for their encouragement, insightful comments and support.

My sincere thanks also to all the academics and researchers including Associate Professor Dr. Sharifah Syed Hassan, Associate Professor Uma Devi, Associate Professor Dr. Rakesh Naidu and Dr. Goh Bey Hing for being part of my work, assessment and thesis. I also appreciate the helps from all the technical officer and staffs in the research and administration offices in Jeffrey Cheah School of Medicine and Health Sciences. Special thanks to my former Honours degree supervisor, Dr. Tam Sheh May for her continuous support, guidance and advices.

I thank my fellow labmates in the Microbiology group; Nurullhudda Zainal, Eng Shu Kee, Vengadesh Letchumanan, Ser Hooi Leng, Loh Teng Hern and Jodi Woan Fei Law for their help and support. Also, to my friends; Jasmine Khairat, Nur Izyani, Athirah Bakhtiar, Shafiq Asnawi, B Saatheeyavaane, Siti Aisyah, Chew Miaw Fang and Christina Injan, thanks for your support and all the fun we had in the past four years.

I would like to express my appreciation to Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia for giving me a PhD scholarship and financial support.

Last but not least, I would like to express my deepest appreciation to all my beloved family members; my husband, Mohd. Faiz Aziim, my daughter, Iman Kamilia, my parents, Azman Haron and Zulaina Rasul, and my siblings, Adzzif Shazreel and Adzziq Shazmeer. Thank you for your presence in our blessed family. I would not have been able to complete this thesis without your continuous love and encouragement.

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ABBREVIATIONS

AMR	Antimicrobial resistance
bp	Base pair
CDC	Centers of diseases control and prevention
DAP	Diaminopimelic acid
DDH	DNA-DNA hybridization
DMK	Desmethylmenaquinones
DNA	Deoxyribonucleic acid
G+C	Guanine + Cytosine
Ha	Hectares
Mbp	Mega base pair
MDR	Multi-drug resistance
MK	Menaquinones
NCDs	Non-communicable diseases
PCR	Polymerase chain reaction
PG	Peptidoglycan
RNA	Ribonucleic acid
RQ	Respiratory quinones
UQ	Ubiquinones
WHO	World Health Organization

Abstract

DISCOVERY OF NOVEL RARE ACTINOBACTERIA ISOLATED FROM MANGROVE ENVIRONMENTS IN THE EAST COAST OF PENINSULAR MALAYSIA

Mangrove forest is a complex ecology and it refers to a group of tropical trees and shrubs that grows in the intertidal zone. It contains various microorganisms including rare actinobacteria that can produce bioactive compounds with interesting bioactivities. The goals of this research were to describe the taxonomic status of novel rare actinobacteria and to screen their bioactivity for antibacterial, neuroprotective and cytotoxic properties. Three rare actinobacteria strains namely MUSC 115^T, MUSC 117^T and MUSC 78^T were successfully isolated from mangrove sediments, collected from Tanjung Lumpur river, Peninsular Malaysia. Characterization using polyphasic approach showed that strains MUSC 115^T and MUSC 117^T have many differences with other species of the genus *Microbacterium* and *Sinomonas,* respectively. Combined phylogenetic, genotypic, chemotaxonomic, and phenotypic analyses proved that strain MUSC 115^T represent a novel species, *Microbacterium mangrovi* sp. nov., while strain MUSC 117^T represent a novel species namely *Sinomonas humi* sp. nov. Meanwhile, strain MUSC 78^T was also characterized by polyphasic approach and results showed that it was different from other genera of the family *Intrasporangiaceae*. Thus, it can be described as a novel genus, *Monashia* gen. nov., the type species of which is *Monashia flava* sp. nov. The successfully characterization of the novel strains have led to the screening of bioactivities on antibacterial, anticancer activities and neuroprotective properties. The crude extract from all novel strains were prepared using solvent extraction method. Antibacterial screening of the crude extracts demonstrated inhibitory effects against 10 bacteria tested including Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, ATCC 70069, ATCC 33591, ATCC BAA-44, *Acinetobacter calcoaceticus* NBRC 13006, *Bacillus subtilis* ATCC 31098, *Pseudomonas aeruginosa* NBRC 112582, *Salmonella typhi*

ATCC 19430, *Vibrio parahaemolyticus* VP103, and *Escherichia coli* ATCC 25922. On the other hand, the neuroprotective studies revealed that *Microbacterium mangrovi* MUSC 115^T extract exhibited significant neuroprotective activity in oxidative stress and dementia model. Meanwhile, the extract of strain *Monashia flava* MUSC 78^T was able to protect the SHSY5Y neuronal cells in hypoxia model. Furthermore, the extracts of *Microbacterium mangrovi* MUSC 115^T and *Monashia flava* MUSC 78^T exhibited anticancer effect against Ca Ski cell line. Based on the findings of this study, mangrove environment is a potentially rich source for the discovery of novel rare actinobacteria with interesting bioactivities for the new drugs development.

Chapter 1

Introduction and general objectives

1.1 Introduction

Soil and water are rich with a wide range of organisms like bacteria and offer an abundant source of potentially novel bacteria. Although many novel and useful bacteria have been identified, 99% of the bacterial species are still unexplored, identified and characterized (Pham and Kim, 2012; Panda *et al.*, 2013). Bacteria such as Actinobacteria have been studied and screened since the 1950s as they are prolific producers of bioactive compounds with different bioactivities such as antibiotics, antifungal, anticancer, antitumor, and immunosuppressive agents (Manivasagan *et al.*, 2014). However, for the past few decades, the rediscovery of the same bioactive compounds from known Actinobacteria had reduced the rate of novel compounds screening activity and development of new drugs (Debbab *et al.*, 2010; Xu *et al.*, 2014). Therefore, most researchers take other alternatives to look for novel Actinobacteria, as well as new bioactive compounds from new or unusual environments such as deserts, deep sea, cave, volcanic, and mangroves. The mangrove forest has gained attention from researchers due to its unique geographical condition located between sea and land, and this unique characteristic offers richness in biological diversity (Xu *et al.*, 2014).

Mangroves are the tidal woodland that occurs in the intertidal zone of sheltered shores, lagoon, estuarine tidal stream, and swamps mudflats of the tropical and subtropical regions of the world (Sengupta *et al.*, 2015). It covers 60% to 75% of the world's tropical and subtropical coastlines (Yang *et al.*, 2013). Of the 75% of global mangroves, 42% can be found in Asia, 20% in Africa, 15% in North and Central America, 12% located in Oceania and the remaining 11% in South America (Giri *et al.*, 2011). Mangrove has unique conditions such as a high salinity, high

moisture, strong wind, high tides, anaerobic condition and muddy soils (Wu and Jiang, 2012; Dissanayake and Chandrasekara, 2014) and contains different types of microorganisms such as bacteria, fungi, protozoa and Actinobacteria.

Actinobacteria are a group of Gram-positive bacteria and have features of both bacteria and fungi (Das *et al.*, 2008), but yet having unique characteristics to restrict them into a different group such as filamentous filaments, germinating spores from hyphae, with multinuclear aerial mycelium, which forms septa at regular intervals, creating a chain of uninucleated spores (de Lima Procópio *et al.*, 2012). Actinobacteria have the largest genomes sizes among prokaryotes; up to 7.7 to 9.0 Mbp (Ventura *et al.*, 2007) and can be divided into two groups; *Streptomyces* and non-*Streptomyces* (also known as rare- Actinobacteria). The genus *Streptomyces* are excellent and continued providers of many different bioactive compounds compared other genera (Sajid *et al.*, 2011). Presently, more than 10, 000 of compounds have been isolated from Actinobacteria where approximately 85% have been obtained from *Streptomyces*. Meanwhile, the remaining 25% bioactive compounds were isolated from rare Actinobacteria (Bérdy, 2005; Bull and Stach, 2007).

The numbers of rare Actinobacteria being discovered are relatively low, as compared to *Streptomyces*, due to the fact that they are difficult to isolate, cultivate and maintain under conventional conditions (Azman *et al.*, 2015). However, there is recent evidence showed that more new genera and species of rare Actinobacteria have been isolated and characterized from various environments including mangrove. As proposed by Hong *et al.* (2009), mangrove environment might become an important and potential source in the discovery of novel rare

Actinobacteria and become an alternative source for new drugs discovery as they have shown a wide range of biological activities.

Potential novel rare Actinobacteria can be characterized by using a modern identification and classification system known as 'polyphasic approach'. The term 'polyphasic approach' was coined by Colwell (1986) and its concept and details were reviewed by Vandammed *et al.* (1996). The polyphasic approach is important in the classification of novel strains as there is no standard phylogenetic method to differentiate between family, genus or species level (Baron, 1996). The polyphasic approach is a combination of different types of information namely genotypic, chemotaxonomic and phenotypic, as well as phylogenetic information. Phylogenetic analysis followed by genotypic methods is the primary analysis in polyphasic approach and the information is derived from nucleic acids analysis (Ramasamy *et al.*, 2014). Meanwhile, chemotaxonomic methods apply analytical methods such as cell wall analysis on the bacterial cells to obtain information on their chemical elements. Phenotypic methods, on the other hand, provide complementary information to genotypic and chemotaxonomic details. It gives morphological, physiological and biochemical features of the microorganism (Rosselló-Mora and Amann, 2001).

The rapid spread of existing infectious diseases, recurrence of old diseases, and developments of new infectious diseases, as well as drug-resistant pathogens, have contributed largely to the urgent global demand to develop new drugs (Davies and Davies, 2010). Besides, an increase in the number of non-communicable diseases (NCDs) and life-threatening diseases such as cancer and neurodegenerative diseases also forces the pharmaceutical industry to look

for the new drugs (Chin and Vora, 2014). Much effort to fight, treat and cure these diseases has been made. However, most of the current drugs and treatments especially synthetic and chemical based drugs have some side effects to the user, and thus new therapeutic agents should be emphasized on natural products, such as from microbial bioactive compounds. Since they are mild and low in toxicity, the side effects can be minimized or eliminated but effective enough to treat the diseases. To provide the demand for new drugs, rare Actinobacteria are excellent resource for the discovery of new bioactive compounds.

Generally, following a discovery of novel bacteria, efforts in screening for potential bioactivity in the novel bacteria will be carried out as some of them may produce different bioactive compounds. Therefore, in this study, the isolation and characterization of novel rare Actinobacteria from mangrove ecosystem are performed as they represent a valuable source for discovering and the study of new species of mangrove Actinobacteria. Furthermore, the screening of bioactive compounds from novel rare Actinobacteria may contribute to the development of new and useful drugs in future to fight against infectious diseases and drug resistant pathogens, as well as different cancer and neurodegenerative diseases.

1.2 General objectives of the study

This study was undertaken with the following objectives:

i. To identify the taxonomic status of novel rare Actinobacteria isolated from mangrove environment using polyphasic approach.

ii. To screen the bioactive compounds extracted from novel rare Actinobacteria for antibacterial and anticancer activities as well as neuroprotective properties.

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Chapter 2

Literature review

2.1 Mangrove in Malaysia

Mangrove forests are considered as one of the most prolific ecosystems in the world and have well-established biological, cultural, and economic importance (Goessens *et al.,* 2014). Mangrove areas cover approximately 60% to 75% of the world's tropical and subtropical coastlines (Azman *et al.,* 2015). In Malaysia, mangroves cover less than 2% of the total land area and occur mainly along the west coast of Peninsular Malaysia, along the east coast of Sabah, and the estuaries of theRejang and Trusan-Lawas rivers of Sarawak. Figure 2.1 shows the distribution of 641, 886 hectares of mangrove forest in Malaysia with 17% of mangroves located in Peninsular Malaysia, 26% in Sarawak and the remaining 57% in Sabah (Abdul Shukor, 2004). In 2006, the total area of mangrove forest in Peninsular Malaysia was estimated at 107, 802 hectares, of which 82, 091 hectares has been designated as Permanent Reserved Forests (PRFs) for production and protection (Jusoff and Taha, 2008).

According to Wieder and Lang (1982), mangrove litter decomposition and the nutrients released is an essential feature of the function of adjacent coastal ecosystems. Soil microorganisms play important roles in the decomposition and mineralization of litter fall which is critical to the productivity of the mangrove ecosystem (Holguin *et al.,* 2001). Microbes constitute the largest pool of metabolic pathways on Earth with potential biotechnological and environment implications. An observation of microbial populations in mangrove soil was done by Ghizelini (2012) where the total microbial biomass was less than 1.2% of the total mass of detritus. In tropical mangroves, bacteria and fungi comprise 91% of the total microbial biomass, whereas 7% is algae and 2% is protozoa (Ghizelini, 2012). The microbial diversity of mangrove

ecosystems provides information on their ecological role and unique biotechnological potential in the field of agriculture, industry, medicine and pharmaceuticals (Lageiro *et al.*, 2007).

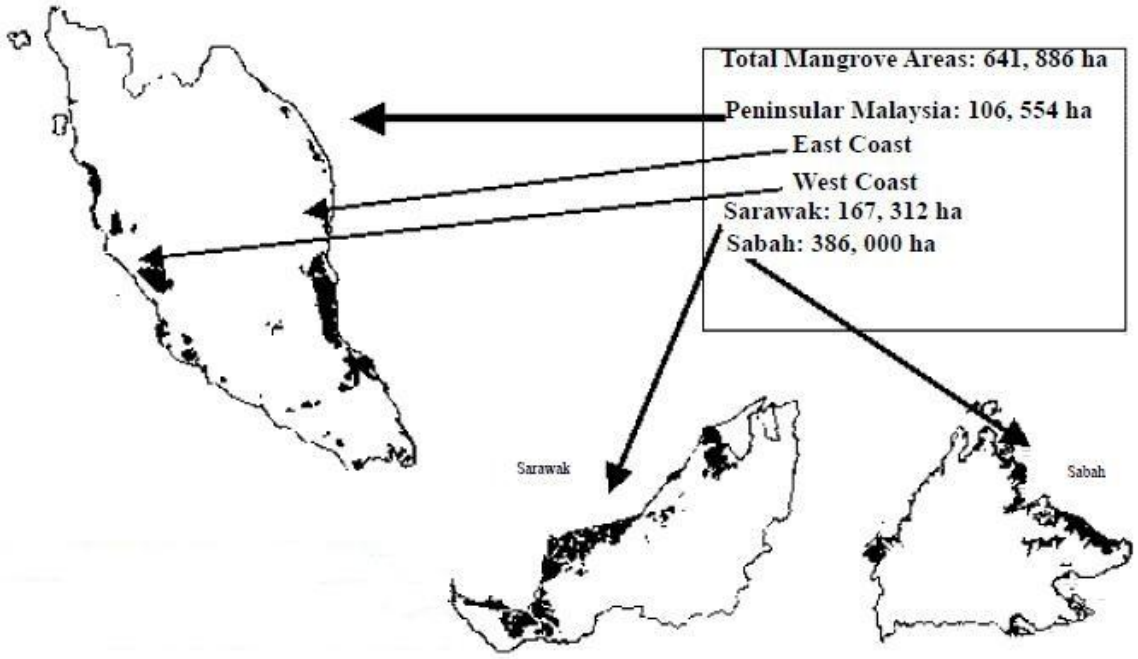

Figure 2.1: Wetland (mangrove and swamp forest) areas in Malaysia (Adapted from Abdul Shukor, 2004)

2.2 Diversity of bacteria in mangroves

Mangrove environment provide a special ecological condition for various bacterial populations. The abundance and activities of bacteria are controlled by various physical and chemical factors. Studies have shown that their action in mangrove is crucial for biogeochemical cycles and transformation of many nutrients (Kathiresan and Bingham, 2001). Bacteria are among the largest providers to carbon flux in sediments. The common bacteria found in mangrove are sulfate-reducing, nitrogen (N_2) -fixing, photosynthetic anoxygenic, phosphate-solubilizing, methanogenic, and enzyme-producing bacteria. The example of bacteria for each

function is shown in Table 2.1. A variety of fungi called “manglicolous fungi” is important for nutrient cycling in mangrove and able to synthesize enzymes such as cellulotyic, ligninolytic, and pectinolytic enzymes. This includes *Cladosporium herbarum, Fusarium moniliforme* and *Cirrenalia basiminuta* (Sahoo and Dhal, 2009).

Table 2.1: Example of bacteria according to their function in mangroves ecosystem

Function	Example of bacteria
Sulfate-reducing	*Desulfotomaculum, Desulfosarcina, Desulfococcus* sp.
N_2-fixing	*Azospirillum, Azotobacter, Rhizobium, Clostridium, Klebsiella*
Photosynthetic anoxygenic	*Chloronema, Chromatium, Beggiatoa, Thiopedia*
Phosphate-solubilizing	*Bacillus, Paenibacillus, Xanthobacter, Vibrio proteolyticus, Enterobacter*
Methanogenic	*Methanoccoides methylutens* sp.
Enzyme-producing bacteria	*Bacillus, Vibrio, Halococcus*

*Adapted with modification from Kathiresan and Bingham (2001).

A number of reports from different geographical locations around the world have described the diversity of bacteria from different mangrove habitats. Table 2.2 summarized the bacterial community found in mangrove ecosystem from different locations. Generally, *Proteobacteria* is a dominant phylum in mangrove followed by *Acidobacteria, Firmicutes* and *Actinobacteria*.

Table 2.2: The bacterial diversity in different mangrove area worldwide

Location	Bacterial community	Reference
Thuwal, Saudi Arabia	*Proteobacteria, Bacteroidetes, Firmicutes, Chloroflexi, Planctomycetes, Actinobacteria*	Alzubaidy *et al.* (2016)
State of Ceará, NE Brazil	*Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, Firmicutes, Planctomycetes, Acidobacteria, Cyanobateria, Verrucommicrobia*	Nogueira *et al.* (2015)
Rantau Abang, Malaysia	*Proteobacteria, Acidobacteria, Firmicutes, Actinobacteria, Nitrospirae, Chloroflexi, Verrucomicrobi, Spirochaetes, Chlamydiae*	Chan and Ismail (2015)
Sundarbans, India	*Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Acidobacteria, Nitrospirae, and Actinobacteria*	Basak *et al.* (2015)
Goa, India	*Proteobacteria, Actinobacteria, Acidobacteria, Clostridia, Anaerolineae, Bacteroidia, Bacilli*	Fernandes *et al.* (2014)
Cananéia, São Paulo State, Southeastern Brazil	*Proteobacteria, Firmicutes, Verrucomicrobia, Chloroflexi, Bacteroidetes, Acidobacteria*	Mendes and Tsai (2014)
Rio de Janeiro and Bahia, Brazil	*Proteobacteria, Firmicutes, and Actinobacteria*	Thomson *et al.* (2013)
Ilha do Cardoso, Brazil	*Proteobacteria, Acidobacteria, Firmicutes, Actinobacteria, Bacteroidetes*	Dias *et al.* (2009)
Futian, China	*Proteobacteria, Cytophaga-Flexibacter-Bacteroides, Actinobacteria, Chloroflexi, Firmicutes, Fusobacteria, Chlamydiae/Verrucomicrobia, Acidobacter, Planctomyces*	Liang *et al.* (2007)

2.2.1 Actinobacteria

Actinobacteria are widely distributed in both aquatic including marine and terrestrial ecosystem. They are Gram positive bacteria with a high G+C content in their DNA, ranging from 51% in some corynebacteria to more than 70% in *Streptomyces* and *Frankia* except for *Tropheryma whipplei;* an obligate pathogen which contained less than 50% G+C (Marco *et al.,* 2007). The name of actinomycete derived from Greek words, 'aktis' (a ray) and 'mykes' meaning fungus based on initial observation of their morphology at that time (Hemashenpagam, 2011).

The phylum *Actinobacteria* is one the most widely distributed and consisted of 6 classes including *Acidimicrobiia, Actinobacteria, Coriobacteriia, Nitriliruptoria, Rubrobacteria,* and *Thermoleophilia* (Bergey's Manual of Systematics of Archaea and Bacteria, 2016). According to recent Bergey's Manual of Systematics of Archaea and Bacteria (2016), there are sixteen orders under the class *Actinobacteria* which are *Actinomycetales* (Goodfellow, 2015a), *Actinopolysporales* (Goodfellow and Trujillo, 2015), *Bifidobacteriales* (Biavati, 2015), *Catenulisporales* (Donadio *et al.,* 2015), *Corynebacteriales* (Goodfellow and Jones, 2015), *Frankiales* (Norman and Benson, 2015), *Glycomycetales* (Labeda, 2015), *Jiangellales* (Tang *et al.,* 2015), *Kineosporiales* (Kämpfer, 2015a), *Micrococcales* (Busse, 2015), *Micromonosporales* (Genilloud, 2015), *Propionibacteriales* (Patrick and McDowell, 2015), *Pseudonocardiales* (Labeda and Goodfellow, 2015), *Streptomycetales (*Kämpfer, 2015b), *Streptosporangiales* (Goodfellow, 2015b) and *Incertae sedis.*

Actinobacteria consist of organisms with different morphologies, from rod-coccoid or coccoid, to permanent or highly differentiated branched mycelium or fragmenting hyphal forms (Atlas, 1997). Actinobacteria have a complex life cycle and display unique development characteristics such as formation of aerial mycelium followed by actinospore, usually in response to nutrient limitation while certain mycobacterium exhibit persistent non-replicating state. The basic morphology of Actinobacteria growing on agar can be seen in Figure 2.2. In common laboratory media, Actinobacteria tend to grow slowly as branching filaments (Brun and Skimkets, 2000).

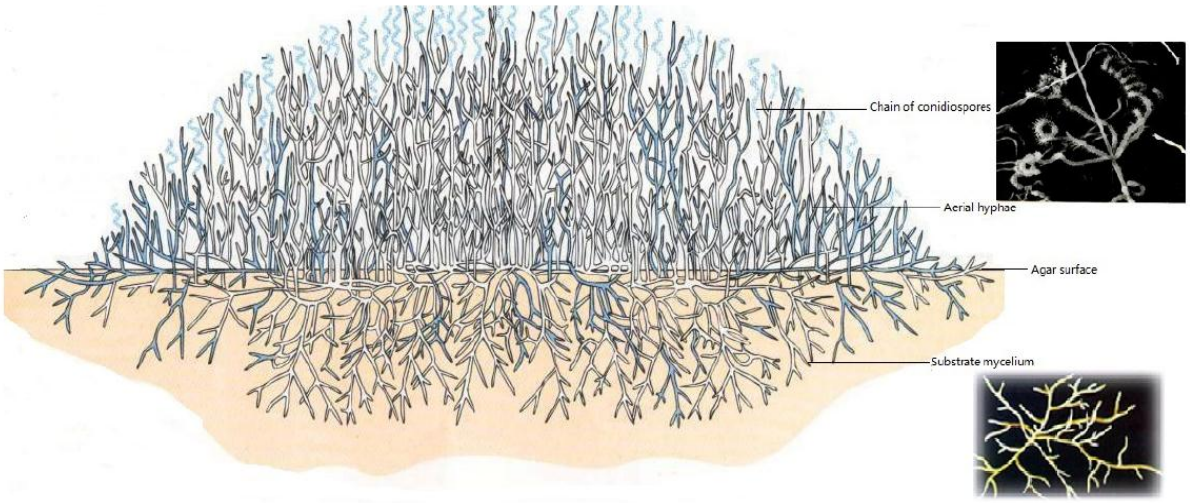

Figure 2.2: Basic morphology of Actinobacteria colony on agar. The Actinobacteria consist of substrate mycelium and aerial mycelium (Adapted from Li *et al.*, 2016).

Actinobacteria can be divided into two main groups; the *Streptomyces*, representing the dominant species in the group; and the non- *Streptomyces* (rare Actinobacteria) (Jose and Jebakumar, 2013). Among other bacteria, Actinobacteria specifically genus *Streptomyces*

possess some of the largest genomes observed up to 8 million base pairs (Mbp) (Õmura *et al.*, 2001). Meanwhile, rare Actinobacteria, for example *Mycobacterium* possess relatively small genomes of about 3.3 Mbp (Akama *et al.,* 2009) and the small genome might possibly a reflection of the fact that they do not undergo complex developmental stage typical of filamentous forms.

Naturally, Actinobacteria are dominant colonizers in soils (Lee *et al.,* 2012) and they are known as saprophyte soil inhabitant (Naik *et al.,* 2013) and important for recycling of refractory biomaterials by humus formation and decomposition (Stach and Bull, 2005). In mangrove ecosystems, Actinobacteria play important roles in mineralization of organic matter, control of mineral nutrients cycle and environmental protection (Pupin and Nahas, 2014). They are important for degradation of cellulose (Pankratov *et al.,* 2006), metal oxidation (Bryan and Johnson, 2008; Johnson *et al.,* 2009), hydrocarbons (Harwati *et al.*, 2007), and nitrate reduction (Van Keulen *et al.,* 2005). Nowadays, mangroves Actinobacteria are of economic importance (Ward and Bora, 2006) as they are a source of antibiotics (Kim *et al.,* 2006; Manivasagan *et al.,* 2009).

2.2.2 Rare Actinobacteria

It has long been recognized that Actinobacteria can be isolated from marine samples (Weyland, 1969). Earlier, the numbers of discovered rare Actinobacteria were low compared to genus *Streptomyces* (Seong *et al.,* 2001). This might due to the fact that they are difficult to isolate, cultivate and maintain under conventional conditions and imitate their original

environment (Jose and Jebakumar, 2013; Azman *et al.,* 2015). However, the number of discovered rare Actinobacteria is increasing, from only 11 genera in 1970 to 100 genera in 2005 and 220 genera by 2010 (Tiwari and Gupta, 2012). Up to January 2016, there are approximately 320 genera of rare Actinobacteria (Euzéby, 1997) isolated from different environments such as deep ocean, desert, mangrove, plants, caves, volcanic rocks and stones. Today, it can be seen that the basic knowledge on the diversity of the habitats, physiology and bioactive compounds of the rare Actinobacteria progressively increased (Tiwari and Gupta, 2012). The numbers of genera presently proved that rare Actinobacteria are widely distributed in the biosphere and their distribution is affected by chemicals, biological and physical elements of the habitat (Hayakawa, 2008).

According to Goodfellow (2010), there are a low number of rare Actinobacteria isolated from marine environments such as the mangrove. Thus, the mangrove environment has gained attention from the researchers looking for novel strains and novel bioactive compound discovery due to its unique condition, where its location between sea and land meant richness in biological diversity (Xu *et al.,* 2014). Recently, there are some studies describing the diversity of rare Actinobacteria from mangrove. Priya *et al.* (2014) discovered three rare Actinobacteria species, namely *Lechevalieria aerocolonies, Lechevalieria flava,* and *Dactylosporangium vinaceum* from Muthuper, Tamil Nadu. Ara *et al.* (2013) found 17 genera of rare Actinobacteria in Dhaka, Bangladesh with *Micromonospora* as the predominant genus. Another study by Karthikeyan *et al.* (2013) reported 14 genera of rare Actinobacteria in Ennoor, east coast of Tamil Nadu, India. In China, six genera of rare Actinobacteria including *Actinomadura, Isoptericola, Microbispora, Nocardia, Nonomuraea,* and *Rhodococcus* were isolated from mangrove soils and plants (Hong

et al., 2009). Meanwhile, *Brevibacterium*, *Dermabacter*, *Kytococcus*, *Microbacterium*, *Nesterenkonia*, and *Rothia* were isolated from mangrove sediments in Brazil (Dias *et al.*,2009). Eccleston *et al.* (2008) reported the occurrence of *Microsmonospora* in Sunshine Coast, Australia while Xie *et al.* (2006) and Huang *et al.* (2008) reported that a rifamycin-producing *Micromonospora* was isolated from mangroves in South China Sea. The discoveries of novel rare Actinobacteria from mangrove habitats, from 2001 until 2015 are listed in Table 2.3.

Table 2.3: Novel mangrove rare Actinobacteria published from year 2001 to 2015

Genus	Strain name and designation	Sources	References
Agromyces	*Agromyces luteolus*, 8^T, *Agromyces rhizospherae*, 14^T, *Agromyces brachium*, 65^T	Rhizosphere of mangroves in the estuary of the Shiira River, Iriomote Island, Japan	Takeuchi and Hatano (2001)
Asanoa	*Asanoa iriomotensis*, TT 97-02^T	Soil at roots of the mangrove *Bruguiera gymnorrhiza*	Tamura and Sakane (2005)
Polymorphospora	*Polymorphospora rubra*, TT 97-42^T	Soil near the roots of *Bruguiera gymnorrhiza* and *Sonneratia alba* at the River Shiira, Iriomote Island, Okinawa, Japan	Tamura *et al.* (2006)
Nonomuraea	*Nonomuraea Maheshkhaliensis*, 16-5-14^T	Mangrove forest in Maheshkhali, Cox's Bazar, Bangladesh	Ara *et al.* (2007)
Micromospora	*Micromonospora pattaloongensis*, TJ2-2^T	Mangrove forest in Pattaloong Province, Thailand	Thawai *et al.* (2008)

Micromonospora	*Micromonospora rifamycinica*, AM105^{T}	Mangrove sediment collected from the South China Sea	Huang *et al.* (2008)
Actinomadura	*Actinomadura maheshkhaliensis*, 13-12(50)^{T}	Mangrove rizhosphere soils of Maheshkhali, Bangladesh	Ara *et al.* (2008)
Verrucosispora	*Verrucosispora lutea*, YIM 013^{T}	Shenzhen Futian Mangrove	Liao *et al.* (2009)
Demequina	*Demequina salsinemoris*, KV-810^{T}	Mangrove soil from a southern island in Japan	Matsumoto *et al.* (2010)
Sphaerisporangium	*Sphaerisporangium krabiense*, A-T 0308^{T}	Tropical mangrove forest soil from Thailand	Suriyachadkun *et al.* (2011)
Isoptericola	*Isoptericola chiayiensis*, 06182M-1^{T}	Mangrove soil from Chiayi Country in Taiwan	Tseng *et al.* (2011)
Micromonospora	*Micromonospora rhizosphaerae*, 211018^{T}	Mangrove *Excocaria agallocha* rhizosphere soil	Wang *et al.* (2011a)
Nonomuraea	*Nonomuraea wenchangensis*, 210417^{T}	Mangrove rhizosphere soil	Wang *et al.* (2011b)
Jishengella	*Jishengella endophytica*, 202201^{T}	*Acanthus illicifolius* root from the mangrove reserve zone in Hainan, China	Xie *et al.* (2011)
Asanoa	*Asanoa hainanensis*, 210121^{T}	Rhizosphere soil of the mangrove fern *Acrostichum speciosum*	Xu *et al.* (2011)
Microbispora	*Microbispora hainanensis*, 211020^{T}	Rhizosphere mangrove soil of *Exoecaria agallocha*, Hainan, China	Xu *et al.* (2012)
Lysinimicrobium	*Lysinimicrobium*	Rhizosphere mangrove	Hamada *et al.*

	mangrovi, HI08-69^{T}	soils from Iriomote Island, Japan	(2012)
Verrucosispora	*Verrucosispora qiuiae*, RtIII47^{T}	Mangrove swamp in Sanya, Hainan Province, China	Xi *et al.* (2012)
Actinomadura	*Actinomadura sediminis*, YIM M 10931 ^{T}	Mangrove sediments from Dugong Creel, Little Andaman, India	He *et al.* (2012)
Agromyces	*Agromyces indicus*, NIO-1018^{T}	Mangrove sediment of the Chorao Island, Goa, India	Dastager *et al.* (2012)
Micromonospora	*Micromonospora haikouensis*, 232617^{T}	Composite mangrove sediment from Haikou, China	Xie *et al.* (2012)
Micromonospora	*Micromonospora maritime*, D10-9-5^{T}	Mangrove soil in Samut Sakhon province, Thailand	Songsumanus *et al.* (2013)
Micromonospora	*Micromonospora avicenniae*, 268506^{T}	Root of *Avicennia marina* collected at mangrove forest in Wengchang, Hainan province, China	Li *et al.* (2013a)
Actinoallomurus	*Actinoallomurus acanthiterrae*, 2614A723^{T}	Rhizosphere soil of mangrove plant *Acanthus ilicifolius* from Touyuan, Wenchang, Hainan province, China	Tang *et al.* (2013)
Micromonospora	*Micromonospora sonneratiae*, 274745^{T}	Root of *Sonneratia apetala* from mangrove forest in Sanya, China.	Li *et al.* (2013b)
Micromonospora	*Micromonospora wenchangensis*,	Composite mangrove soil from Wenchang, Hainan	Ren *et al.* (2013)

	2602GPT1-05^T	province, China	
Microbacterium	*Microbacterium mangrovi*, MUSC 115^T	Mangrove soil of Tanjung Lumpur river, State of Pahang, Malaysia	Lee *et al.* (2014a)
Mumia	*Mumia flava*, MUSC 201^T	Mangrove soil of Tanjung Lumpur river, State of Pahang, Malaysia	Lee *et al.* (2014b)
Sinomonas	*Sinomonas humi*, MUSC 117^T	Mangrove soil of Tanjung Lumpur river, State of Pahang, Malaysia	Lee *et al.* (2015)

2.3 Polyphasic approach: a consensus approach to bacterial systematics

Microbial taxonomy or microbial systematics refers to the classification, identification and nomenclature of microorganisms (Sarethy *et al.,* 2014). Taxonomy and systematics are frequently used interchangeably but both differ in their meaning. Taxonomy refers to the theory and practice of classifying organisms while systematics study the diversity of organisms including their phylogeny and all potential biological interaction (Prakash *et al.*,2007).

Previously, conventional microbiology methods classify bacteria based on their morphology, physiology and biochemistry. Most traditional descriptions were based on few characteristics, such as colour, shape, size, staining properties, host-range, motility, pathogenicity, and use of carbon sources (Clarridge, 2004). This may lead to unclear taxonomic status because the information is insufficient to be used for bacterial systematics. Therefore, bacterial classification should be based on more practical technique and method, using several

approaches for the full characterization of bacteria. The first attempt to study microbial taxonomy was based on single-stranded DNA (Schildkraut *et al.,* 1961). This was a major step classifying microbe towards the polyphasic system development. Using genotypic, chemotaxonomy and phenotypic methods to determine the microbial taxonomic status is known as the 'polyphasic approach'. This term was coined by Colwell (1986) and the concept and details were reviewed by Vandamme *et al.* (1996).

Generally, a polyphasic approach is proposed to determine the taxa below species level (Ramasamy *et al.,* 2014). Ideally, the characterization of a novel bacterial species should be sufficiently comprehensive to differentiate the members of the newly described taxon from related taxa, thus avoiding lack of species descriptions that contribute to the uncertainty of classification. In polyphasic approach, a range of genotypic and phenotypic procedures are recommended as minimal standards, although these requirements vary according to taxa. Nevertheless, it is clear that recommended analysis should be based their taxonomic rank (Table 2.4). It is also important that the description of new taxa must be consistent with the phylogenetic data from 16S rRNA sequencing (Stackebrandt *et al.,* 2002).

The application of polyphasic approach has led to profound changes and improvements in bacterial systematics of mainly important industrial groups such as the Actinobacteria. The comprehensive polyphasic approach enabled the description of novel rare Actinobacteria genera, notably *Barrientosiimonas humi* (Lee *et al.,* 2013) and *Branchiibius hedensis* (Sugimoto *et al.,* 2011). Likewise, the novel Actinobacteria species, for example, *Leifsonia pindariensis* (Reddy *et al.,* 2008), *Gordonia cholesterolivorans* (Drzyzga *et al.,* 2009) and many more have been

successfully described using polyphasic approach. The polyphasic approach is also used for identification of novel yeast (Aveskamp *et al.*, 2010) and fungus (Sipiczki and Kajdacsi, 2009).

Table 2.4: Methods used in microbial systematics based on their taxonomic rank.

Cell component	Analysis	Taxonomic rank		
		Genus	Species	Subspecies
DNA	Base composition G+C%	√	√	
	DNA-DNA hybridization		√	√
	Restriction pattern (RFLP, ribotyping)		√	√
	Next generation sequencing (NGS)	√	√	√
DNA fragments	DNA probes	√	√	√
	DNA sequencing	√	√	√
	Multilocus sequencing		√	√
	DNA fingerprinting		√	√
Ribosomal RNA	DNA-rRNA hybridization	√	√	
	Sequencing	√	√	
Proteins	Amino acid sequence	√	√	
	Multilocus enzyme electrophoresis	√		√
	Serology	√	√	√
Chemotaxonomy	Peptidoglycan	√		
	Fatty acids	√	√	
	Polar lipids	√	√	
	Isoprenoid quinones	√	√	
	Mycolic acids	√	√	
	Polyamines	√		
	Teichoic acids	√	√	
Whole cell	Pyrolysis mass spectrometry		√	√
	MALDI biotyping		√	√
	Rapid enzyme tests		√	√
Expressed	Morphology	√	√	
Characters	Physiology	√	√	

*Adapted with modification from Goodfellow (2000), Bull *et al.* (2000), Moore *et al.* (2010) and Chun and Rainey (2014).

2.3.1 Genotypic characterization

Modern taxonomy involving DNA and RNA such as DNA-DNA hybridization, DNA G+C content and 16S rRNA gene sequence analysis are now commonly used in bacterial taxonomy (Stackebrandt and Ebers, 2006; Tindall *et al.*,2010).

2.3.1.1 16S rRNA gene sequence

Studies on bacterial identification shows that phylogenetic assessment based on conserved sequence of genome is more stable compared to the classification based on phenotypic traits and other characterizations. In 1965, Dubnau *et al.* described the conservation of 16S rRNA gene sequence in *Bacillus*. It is then extensively used in bacterial taxonomy after the pioneering work of Woese (1987) where the phylogenetic relationships of bacteria, and, indeed, all life-forms, could be identified by comparing a stable part of genetic code. The length of 16S rRNA gene is approximately 1,550 base pairs (bp) and it offers a unique advantage to understand the phylogeny of taxa studied. It is highly conserved in prokaryotes which allow the design of universal primers and utilization in determining the relationships between taxa at higher levels whereas more variable regions allow discrimination between closely related taxa (Monciardini *et al.*, 2002).

Theoretically, 97% of 16S rRNA gene homology match to the 70% DNA-DNA hybridization (DDH) threshold (Stackebrandt and Goebel, 1994). In many cases, new bacteria are considered as members of the same species if they shared 97% or more of 16S rRNA gene

sequence similarity (Tindall *et al.*, 2010; Zhi *et al.*, 2012; Oren and Garrity, 2014). Many studies were done to standardize "cutoff similarity value" that can be used to differentiate higher taxa since (Stackebrandt and Ebers, 2006; Oren, 2010; Zhi *et al.*, 2012; Yarza *et al.*, 2014). Table 2.5 summarized the recognized standards of taxonomic threshold commonly used to identify bacteria systematics based on 16S rRNA sequence similarity.

Table 2.5: Taxonomic thresholds of bacteria

	Genus	Family	Order	Class	Phylum
Minimum sequence identity (%)	94.8	87.65	83.55	80.38	77.43
Threshold sequence identity (%)	94.5	86.5	82.0	78.5	75.0

*Adapted with modification from Yarza *et al.* (2014)

2.3.1.2 DNA-DNA hydridization (DDH)

DNA-DNA hybridization or DDH was introduced into prokaryotes systematics in 1960s. This technique is based on a comparison between whole genome of two or more bacterial species and is considered as the 'gold standard' criteria for species delineation of bacteria (Wayne *et al.*, 1987). Usually, DDH is used when the new taxon contains more than one strain, in order to prove that all members in the same taxon have a high degree of hybridization among each other (Richter and Rosselló-Móra, 2009). DDH is needed when strains share more than 97% of 16S rRNA gene sequence similarity (Figure 2.3) and comparison must be performed with all related type strains to confirm that there are adequate variation to support the classification of the strain as new taxon (Stackebrandt and Ebers, 2006).

Since 1970s, bacterial species have been described based on information from DDH experiments (Ramasamy *et al.,* 2014). In this analysis, the whole genetic similarity between strains is evaluated by the degree to which their genomes hybridize under standardized conditions. Isolates that show more than 70% DNA-DNA binding values and less than 5% differences in their melting temperature are considered to belong to the same species. Meanwhile, strains that share less than 70% DNA-DNA binding values definitely belong to distinct species (Gevers *et al.,* 2005; Ramasamy *et al.,* 2014).

2.3.1.3 DNA G+C content

The genomic DNA G+C content can be defined as the ratio of cytosine and guanines within the total number of nucleotides in the genomes. Previous study showed that bacterial genomic G+C content ranges from 25% to 75% (Wu *et al.,* 2012). The G+C ratio is one of the traditional genotyping techniques in bacteria systematics and it commonly used in taxonomic study of bacteria (Tindall *et al.,* 2010; Mesbah *et al.,* 2011) such as phylum Actinobacteria which always referred to the high G+C content Gram positive bacteria. In genotypic characterization, the difference in the percent G+C content is more than 5% and more than 10% between strains were used to classify them within distinct species or genera, respectively (Goodfellow *et al.,* 1997). However, such values are not applied to all bacterial genera, for example all species within the genus *Rickettsia* exhibit less than 5% difference in DNA G+C content (Fournier and Raoult, 2009). Moreover, this analysis is an important requirement for determining the conditions used in DDH.

Traditionally, G+C contents were estimated using thermal denaturation, buoyant density in Caesium chloride (CsCl) or melting profiles. Meanwhile, modern techniques rely on HPLC and real-time PCR (Meier-Kolthoff *et al.,* 2014). An advanse in DNA sequencing technique however has enabled us to estimate the amount of G+C contents directly from completely sequenced genomes. The high coverage obtained with modern sequencing techniques yields high sequence accuracy (Mavromatis *et al.,* 2012). Previous data showed that the G+C content in within-species variation is at most 3% (Mesbah *et al.,* 2011) or 5% (Rosselló-Mora and Amann, 2001).

2.3.1.4 DNA-based typing methods: Repetitive PCR

The introduction of the PCR methodology into the microbiology laboratory has opened a vast array of applications. PCR-based DNA-typing methods attracted much interest because of their universal applicability, simplicity, rapidity, and high discriminatory power (Prakash *et al.,* 2007). The methods include pulsed-field gel electrophoresis (PFGE), ribotyping, restriction fragment length polymorphic DNA assay (RAPD), arbitrary primed PCR (AP-PCR), amplified fragment length polymorphism (AFLP), and repetitive element based PCR (rep-PCR).

Rep-PCR is a method for fingerprinting bacterial genomes, which examines strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. This method can identify the species of interest to species, subspecies, and strain levels (Versalovic *et al.,* 1994; Prakash *et al.,* 2007). There are three type of repetitive

elements used for typing purpose known as Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic consensus sequence (ERIC) and BOX elements.

The BOX element found in *Streptococcus pneumoniae* is an inversely repeated DNA element that potentially forms stable stem-loop structure and has been shown to be transcribed in *S. pneumonia* (Croucher *et al.,* 2011). Box sequences are highly conserved repetitive DNA elements found in most Gram positive bacteria (Martin *et al.,*1992) and one Gram-negative bacteria, *Pseudomonas* (Marques *et al.* 2008; Javadi *et al.,*2014). Moreover, a BOX element does not share any sequence homology either with REP or ERIC (Olive and Bean, 1999). Due to the characteristics of BOX element, it has been used for genotyping in discovery of novel Actinobacteria such as *Bifidobacterium* (Masco *et al.,* 2003) and *Streptomyces* (Anita *et al.,* 2007).

2.3.2 Chemotaxonomic characterization

In polyphasic approach, chemotaxonomic characterization can be defined as an analytical way to collect information on different chemical components of chemotaxonomic markers in bacterial cells to group or organize them into different taxonomic ranks (Rosselló-Mora and Amann, 2001). The principle of chemotaxonomy is based on uneven distribution of these markers among different microbial groups and the use of these analytical methods varies between groups. The most commonly used chemical markers are shown in Table 2.6.

Table 2.6: Markers used in polyphasic approach

Site in cell	Composition
Cell	Sugars
Cell wall	Amino acid
Plasma membranes	Polar lipid, quinones, fatty acids, mycolic acids

*Adapted from Wang and Jiang (2016).

2.3.2.1 Cell wall analysis

The cell walls of Actinobacteria were found to be made of sugars, amino sugars and amino acids, in addition of glucosamine and muramic acid of peptidoglycan (PG) (Cummins and Harris, 1958). Generally, 2, 6-diaminopimelic acid (DAP) is commonly distributed as a main amino acid in bacteria. The presence of DAP isomers is one of the important information concerning the cell wall PG of Gram positive bacteria including Actinobacteria. Usually, the DAP is located in the cell wall of the Gram positive bacteria contain PG with one of the DAP isomers.

The identification of DAP is the earlier step of cell wall analysis in Gram positive bacteria. However, further amino acid analysis will be performed if no DAP is detected in the cell using purified cell wall fraction. The presence of DAP in bacteria including Actinobacteria is in either *meso*-DAP or LL-DAP form, however, only genera *Kitasatosporia* contained both *meso*- and LL-DAPs (Omura *et al.,* 1982). *Meso*-DAP can be found in the cells of vegetative and filamentous mycelia while LL-DAP are located in the submerged and aerial spores (Takahashi *et al.,* 1984). Another type of DAP known as 3-hydroxy-DAP is found in certain genera of

Actinobacteria such as *Actinoplanes* and *Ampullariella*,, and usually it is found together with *meso*-DAP.

2.3.2.2 Whole cell sugar analysis

Result from analysis of sugars of whole cells is widely used for the description of Gram positive bacteria including Actinobacteria (Busse *et al.*, 1996). The whole cell sugar arrangement divided Actinobacteria into 4 types as shown in Table 2.7 (Lechevalier and Lechevalier, 1970). However, Labeda (1987) described another group of the whole cell sugar which consisted of rhamnose and galactose, making a total of 5 types. The presence of madurose in type B is one of the important markers to differentiate certain genera of Actinobacteria such as *Actinomadura, Streptosporangium* and *Microbispora* from other Actinobacteria. Lechevalier and Lechevalier (1980) introduced the cell wall chemotype of Actinobacteria based on the components in the cell wall which consist of whole cell sugars and amino acids (Table 2.8).

Table 2.7: Whole cell sugar patterns of Actinobacteria.

Pattern	Characteristic sugars
A	Arabinose, galactose
B	Madurose
C	No sugar
D	Arabinose, xylose
E	Rhamnose, galactose

*Adapted from Lachevalier and Lechevalier (1970), Labeda (1987) and Wang and Jiang (2016).

Table 2.8: The cell wall chemotype in Actinobacteria.

Cell wall chemotype	Characteristic cell wall composition
I	$_{LL}$-DAP, glycine, no sugar
II	*meso*-DAP, glycine, arabinose, xylose
III	*meso*-DAP, madurose
IV	*meso*-DAP, arabinose, galactose, mycolic acid
V	Lysine, ornithine
VI	Variable presence of lysine, aspartic acid, galactose
VII	Diaminobutyric acid, glycine
VIII	Ornithine
IX	*meso*-DAP, various amino acids
X	*meso*-DAP, $_{LL}$-DAP

*Adapted with modification from Lachevalier and Lechevalier (1970, 1980), Wellington *et al.*, 1992), Bora (2015) and Wang and Jiang (2016).

2.3.2.3 Phospholipids

Phospholipids are the major structural components of membranes. It appears in bilayer configuration in cell membrane of bacteria as well as membrane structure of eukaryotes. Phospholipids in the bacterial membrane play important roles such as permeability barrier, provision of the environment for many enzyme and transporter proteins, and they influence membrane related process such as protein export and DNA replication (Cronan, 2003). Bacterial phospholipids consist of two components; a polar, hydrophilic head and a hydrophobic tail, thus they show amphipathic characteristic (Mrozik *et al.,* 2004). Phospholipids are differentiated based on the charge and polar head group. Differences in polar head group provide important information for the classification of Actinobacteria. Table 2.9 shows the major types of phospholipids that are commonly found in Actinobacteria.

Table 2.9: Different types of phospholipids in Actinobacteria.

Charge	Polar head group	Name (abbreviation)
1+	Glycerol	Phosphatidylglycerol (PG)
1+	Acylated mannosylinositols	Phosphatidylinsitol mannosides (PIM)
1+	Butane-2,3-diol	Phosphatidylbutanediol (PB)
1+	Inositol	Phosphatidylinositol (PI)
2+	Phosphatidylglycerol	Diphosphatidylglycerol (DPG)
0	Ethanolamine	Phosphatidylethanolamine (PE)
0	Choline	Phosphatidylcholine (PC)
0	Methylethanolamine	Phosphatidylmethylethanolamine (PME)

*Adapted with modification form Minnikin and O'Donnell (1984) and Bora (2015).

The analysis of phospholipid composition in Actinobacteria has been described by Lechevalier and Lechevalier (1980). They distinguished five major patterns of phospholipids in Actinobacteria as shown in Table 2.10. The phospholipid pattern is important in Actinobacteria classification to differentiate between some genera, such as *Amycolata* and *Amycolatopsis*, which have the same common morphology and cell wall content but different phospholipid pattern (Labeda, 1987). Lechevalier *et al.* (1977) showed that the common type of phospholipid composition in non-motile actinomycetes was type PII. Meanwhile, Hasegawa *et al.* (1979) showed that in motile actinomycetes including members of genera *Kitasatoa, Amorphosporangium, Ampullariella, Actinoplanes, Actinosynnema,* and *Geodermatophilus* have phospholipid type PII, where it contained major amounts of phosphatidylethanolamine (PE). Suzuki *et al*. (1993) however stated that most of Actinobacteria contained phosphatidylinositol.

Table 2.10: Classification of phospholipids in Actinobacteria.

Group	Phospholipids
I	Phosphatidylglycerol with phosphatidylinositol, phosphatidylinositol mannosides
II	Phosphatidylethanolamine
III	Phosphatidylcholine, phosphatidylethanolamine, phosphatidylmethylethanolamine and phosphatidyglycerol
IV	Phosphatidylethanolamine and phosphatidylmethylethanolamine
V	Phosphatidylethanolamine, and phosphatidylglycerol with phosphatidylethanolamine variable

*Adapted with modification after Lechevalier *et al.* (1977), Labeda (1987), Wang and Jiang (2016).

2.3.2.4 Peptidoglycan

Another important component of the bacteria's cell wall besides fatty acids is peptidoglycan (PG). PG or also known as murein is a continuous covalent macromolecular structure that can be found exclusively on the outside of the cytoplasmic membrane in most of bacteria (Schleifer and Kandler, 1972; Nanninga, 1998). The chemical structure of PG contains amino-sugar backbones with four amino acids joined by peptide bone chains and a diamino acid. These chains are cross-linked either through or directly with an interpeptide bridge of L-amino acid residue between the diamino acid and the D-Ala residue at position four on an adjacent chain (Vollmer *et al.,* 2008). The amino-sugar backbones are the main structure of the PG in all bacteria. However, the variation between the taxa may exist due to the different type diamino acid of the tetrapeptide chain and the composition of the interpeptide bridge. This variation is important information for Gram-positive bacteria (Vollmer *et al.*, 2008).

Generally, the PG of bacteria can be divided into two groups; type A and type B. The main difference between these groups is the cross-linked position of the peptide subunit. The summary of the type of PG in bacteria is shown is Table 2.11. In Actinobacteria, the major PG group found is type A and *Streptomyces* contained A1γ PG (Schleifer and Kandler, 1972). Furthermore, the muramic acid found in bacteria is either type A, which is N-acetylmuramic acid, or type B, N-glycolylmuramic acid (Uchida and Aida, 1977; Uchida and Aida, 1984).

2.3.2.5 Quinones

Analysis of respiratory quinoness (RQ) structure is one of the most extensive used culture-independent techniques for characterizing the microbial community structure (Hu *et al.*, 1999), as the analysis offers new insight into the composition of the microbial communities and their abundance. Quinones are lipid-soluble substances distributed in nature (Vandamme *et al.*, 1996) and they can be found in prokaryotic cells (Hiraishi, 1988).

Table 2.11: Types of peptidoglycan in bacteria

Type A: Cross-linked positions 3 and 4		
A1	Direct – no interpeptide bridge	
	A1α	L-Lysine
	A1β	L-Ornithine
	A1γ	*meso*-DAP
A2	Polymerized peptide bridge	
	A2α	L-Lysine
A3	Monocarboxylic acid and/or glycine	
	A3α	L-Lysine
	A3β	L-Ornithine
	A3γ	$_{LL}$-DAP
A4	Dicarboxylic acid	
	A4α	L-Lysine
	A4β	L-Ornithine
	A4γ	*meso*-DAP
	A4δ	L-DAB
A5	Dicarboxylic acid and lysine interpeptide bridge	
Type B: Cross-linked positions 2 and 4		
B1	L-diamino acid	
	B1α	L-Lysine
	B1β	L-Homoserine
	B1γ	L-Glutamic acid
	B1δ	L-Alanine
B2	D-diamino acid	
	B2α	L-Ornithine
	B2β	L-Homoserine
	B2γ	L-DAB

*Adapted with modification after Schleifer and Kandler (1972), Schleifer and Stackebrandt (1983), and Schleifer (1985).

The common quinones found in bacteria is ubiquinones, UQ, (1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone) or known as co-enzyme Q and menaquinones, MK, (1-isoprenyl-2-methyl-naphtho-quinone), which can be found in the bacterial plasma membrane as they participate in electron transport in respiratory chains (Hiraishi, 1999). According to Hedrick and White (1986), MK is commonly found in Gram-negative, Gram-positive bacteria, and archaebacteria while UQ are commonly found in eukaryotes but also present in some gram-negative bacteria. Other type of quinone found in bacteria is desmethylmenaquinones (DMK). However, DMK are not so widely distributed and commonly reported in some pathogenic enterobacteria and *Streptococcus feacalis* (Collins and Jones, 1981). The chemical structure of UQ and MK are shown in Figure 2.3. The differences of UQ and MK can be seen at the polyprenyl side chains which consist of different number of isoprene units and in the degree of saturation. The difference in this chain is the foundation in bacterial taxonomy (Hanif *et al.*, 2012).

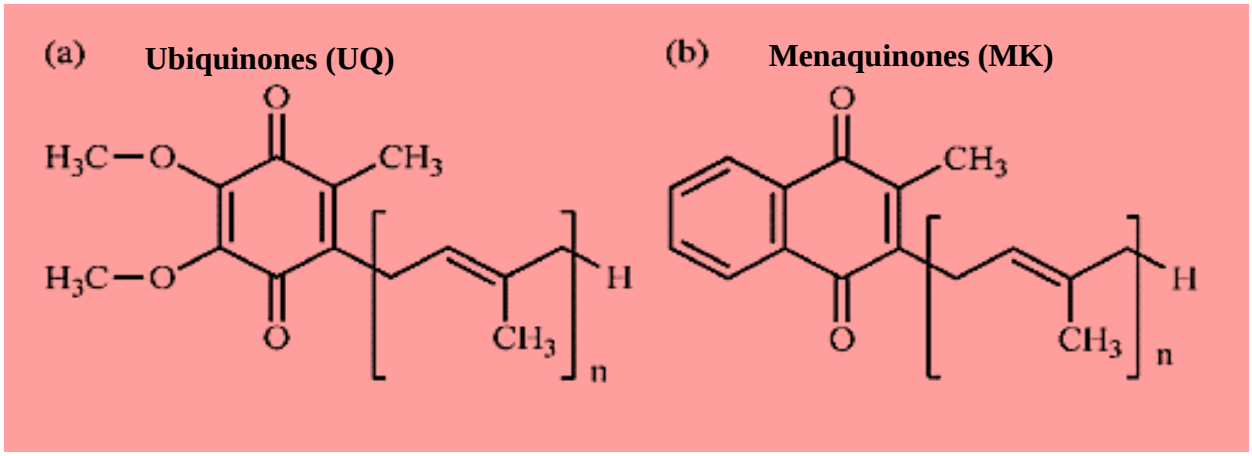

Figure 2.3: Chemical structure of UQ and MK. n is the length of the isoprene of the side chain.

According to Batrakov and Bergelson (1978), the first indication for the presence of menaquinones in streptomycetes cells was reported by Lester and Ctane in 1959. They discovered the menaquinone in *Streptomyces griseus* but were unable to explain the structure. Comparing the MK in other microorganisms, it is more highly saturated in certain Actinobacteria so that they are assumed evolutionary to be the most ancient type of quinones (Schoepp-Cothenet *et al.,* 2009). Moreover, the presence of partially hydrogenated MK in most of the Actinobacteria species usually is more than 20% of the total quinones composition, thus differentiating Actinobacteria from other bacteria (Hiraishi, 1999).

2.3.2.6 Fatty acids

Fatty acids are one of the most important elements of cellular materials as important sources of metabolic energy in all organisms (Fujita *et al.*, 2007). It can be found in simple structures between 8 and 20 carbon atoms. Fatty acids are located in the cytoplasmic membrane and lipopolysaccharides of the outer membrane cell of Gram negative bacteria as well as lipoteichoic acids in Gram positive bacteria (Wang and Jiang, 2016).

The biosynthesis of fatty acids is the initial stage in the development of membrane lipids and is important for all cells except in Archea. Based on the biosynthetic relationships, there are three major types of membrane fatty acids known as straight-chain (including mono-unsaturated acids), branched-chain, and complex fatty acid (Table 2.12) (Bora, 2015). Straight-chain fatty acid family is commonly found in bacteria while branched-chain fatty acid, β-OH and cyclopropane are not common but still very significant (Lechevalier, 1989; Zelles, 1999). In

Actinobacteria, the common fatty acids are 10-Methyloctadecanoic acids and its homologs (Kroppenstedt, 1985; Embley *et al.*, 1987). The information on the carbon chain length, the presence of saturated or unsaturated, occurrence of methyl groups fatty acids, cyclopropane fatty acids and hydroxyl-fatty acid with an OH-group at position 2 or 3 of the molecule are used in taxonomy analysis.

Table 2.12: Major type of fatty acids found in bacteria

Type		Fatty acids
1	Straight chain fatty acids	
	1a	Saturated and unsaturated straight chain
	1b	10-methyl branched fatty acids
	1c	Cyclopropane fatty acids
2	Branched fatty acids	Iso- and anteiso-fatty acids
3	Complex branched fatty acids	Iso-, anteiso- and 10-methyl branched fatty acids

*Adapted from Bora (2015).

2.3.3 Phenotypic characterization

Phenotypic characterization is a fundamental analysis of microbial description and characterization. It is one of the important methods for differentiation of taxa at the highest to lowest levels from phylum to species and subspecies. Phenotypic characterization, the examination of morphological, physiological and biochemical characterization, is the oldest tool for the description and classification of bacteria (Moore *et al.*, 2010).

The morphology of an organism is usually based on colony characteristics such as shape, size, elevation, opacity, pigmentation, colour, production of slime as well as motility on solid surfaces and odor. The growth conditions such as aerobic, anaerobic or carbon dioxide (CO_2) requirement and different types of media are also employed under phenotypic analysis (Tindall *et al.*, 2010). Moreover, features of the cell including size, shape, cell arrangements, Gram reaction, production of spores, capsule, flagella and motility are described using light microscopy. However, electron microscopy is recommended for high resolution images to observe the spore, flagella and cell (Li *et al.*, 2016).

The physiological and biochemical characteristics of the microbe can be described in various and certain range of conditions such as temperature, pH, atmospheric conditions, salinity, effect of growth in presence of antimicrobial compounds, utilization of carbon, nitrogen and chemical sources, and production of different enzymes (Tindall *et al.*,2010). Overall, phenotypic characterization can now be easily examined and is a straight forward approach as compared to genotypic and chemotaxonomic characterization.

Actinobacteria systematics is providing a taxonomic road map to genes hence products, including the discovery of first-in-class drug candidates (Bull and Statch, 2007; Kumar and Goodfellow, 2008). To date, most of the identified microbial metabolites including those from Actinobacteria are used as antibacterial, anticancer, antifungal, antiviral, herbicides and insecticidal agents.

2.4 Actinobacteria bioactive compounds

All organisms biosynthesize an enormous amounts of organic compounds to functional end products to grow, live, and reproduce. Natural products, generally secondary metabolites, are any element produced by living organisms such as plants (Veeresham, 2012) and microbes (Genilloud, 2014). It presents an alternative way to address the urgent need for new drugs due to their low production cost, novelty and structural diversity. Microbes have been proved as the most promising sources of natural products and have played an important role in organic chemistry and drug discovery (Bull, 2004; Solanki *et al.,* 2008).

Secondary metabolites are defined as compounds with a low molecular weight with the size of less than 1500 Daltons and they are not the products of the primary metabolic pathways (Keller *et al.,* 2005). Usually, secondary metabolites can be metabolic intermediates or end products from complicated and often lengthy biosynthetic pathways. Basically, the production of these secondary metabolites is controlled by specific genes that regulate the onset of secondary production (Hodgson, 2000). Actinobacteria, mainly genus *Streptomyces* has ability to produce variety of secondary metabolites as bioactive compound such as antibiotics due to the function of the gene non-ribosomal polyketides synthase (NPRS) and polyketide synthase (PKS) pathway (Salomon *et al.,* 2004).

Along with the different types of microbe, Actinobacteria play an importance role in the field of biotechnology, as producers of many bioactive compounds with industrial, medical, pharmaceutical and agricultural applications (Janardhan *et al.,* 2014). About 23, 000 antibiotics

have been discovered, approximately 10, 000 of them were isolated form Actinobacteria (Manivasagan *et al.*,2013) specifically from the genus of *Streptomyces*, which are prolific and an excellent producer of bioactive compounds (Subramani and Aalbersberg, 2012). Actinomycin is the first antibiotic discovered in an Actinobacteria known as *Streptomyces antibioticus* in 1940, followed by streptothricin from *Streptomyces lavendulae* in 1942 and streptomycin from *Streptomyces griseus* in 1944 (Waksman, 1953). *Streptomyces* have been the major source of clinical antibiotics and are responsible for over 75% of all antibiotics while the remaining 25% of the compounds are isolated from rare Actinobacteria (Berdy, 2005). Besides antibiotics, Actinobacteria also are able to produce other bioactive compounds with activity such as antimicrobial, anticancer, antitumor, enzyme inhibitors and immunosuppressive agents (Bibb, 2005; Manivasagan *et al.*, 2013).

Despite of the great success of the previous screenings for useful bioactive compounds, the chance of finding new bioactive compounds from *Streptomyces* has decreased, but the re-isolation of the known compound has increased (Hong *et al.*,2009; Hamedi *et al.*,2015). At the same time, the development of multidrug-resistant (MDR) pathogens and new diseases are becoming a serious threat to public health (Ekwenye and Kazi, 2007; Xiong *et al.*, 2011). The current shortage of drug against MDR pathogens and other deadly diseases caused the dramatic increase in demand to look for new bioactive compounds for the development of new drugs (Wright and Sutherland, 2007; Neha and Sandeep, 2014) from other groups such as rare Actinobacteria.

2.4.1 The urgent need to develop new drugs

An important reason for discovering new bioactive compounds is to defeat the problem of infectious diseases (Talbot *et al.*, 2006), antibiotic resistant pathogens (Payne *et al.*, 2007) and to combat non-communicable diseases (NCDs) such as cancer (Olano *et al.*, 2009) which are continuously developing resistance to existing drugs (Ekwenye and Kazi, 2007). Previously, infectious diseases commonly occurred in developing countries while the chronic diseases such as cancer were mostly found in high income countries. However, the pattern of the diseases are changing with the infectious diseases remain as a main problem in most of the countries and chronic diseases including cancer is now become the major cause of death worldwide (U.S. Department of State, 2016). According to World Health Organization (WHO), in 2012, NCDs were responsible for 68% of all death globally while 23% were from communicable diseases including infectious diseases (World Health Organization [WHO], 2014). In 2013, infectious diseases, cancer and neurodegenerative diseases are in the top 10 for leading causes of death (Centers of Diseases Control and Prevention [CDC], 2014).

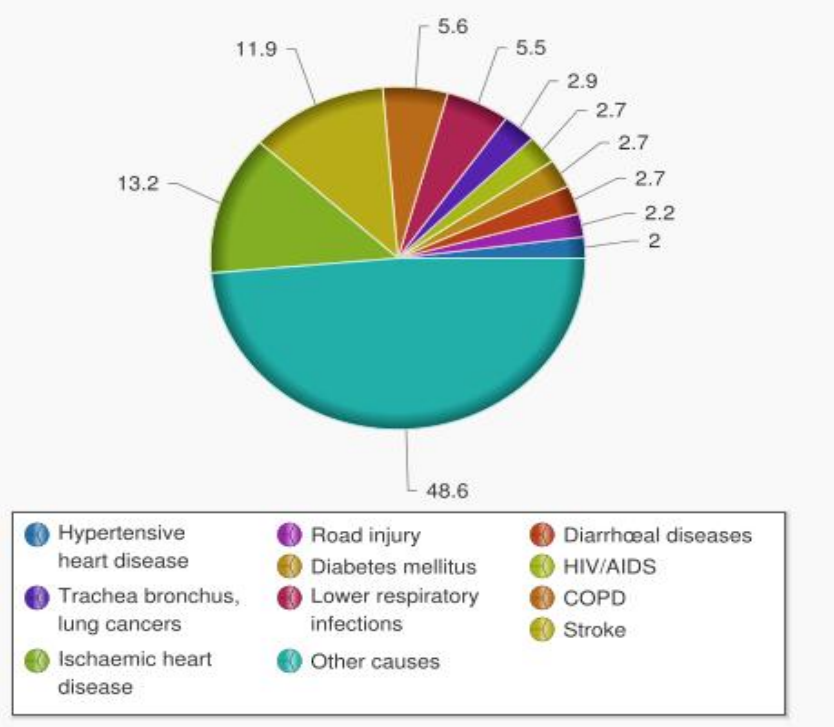

Figure 2.4: The top 10 leading causes of death in the world by percentages (Adapted from WHO, 2014).

2.4.2 Infectious diseases

Earlier in 1900, infectious diseases were the leading cause of human death in the world (Yoneyama and Katsumata, 2006). However, in recent years, it became the second major cause of death globally and third in developed countries (WHO, 2004). Infections from antimicrobial resistance (AMR) bacteria are now become common cases and some of them are resistant to multiple types of antibiotics (CDC, 2014). An AMR occurs as a part of a natural evolution process which means the efficiency of antimicrobials is limited (Michael *et al.*, 2014). The resistance process can be significantly slowed but not stopped. However, overuse and misuse of antimicrobial in humans and animals is accelerating the process. Overuse and misuse of the

antimicrobial caused not only AMR (Hayek *et al.*, 2013) but also decreased human immunity, imbalance the internal flora which leads to endogenous infections and mutation in microbes in order for them to adapt with their new condition (Song and Zheng, 2015).

According to CDC (2014), one of the major actions to fight the spread of drug resistant bacteria is to encourage the development of new antibiotics and new diagnostic tests. The increasing number of resistant microbes today resulted in the high demand of the new antibiotics. If there are no new drugs discovered, most of current treatment and therapies will not effectively work in future even for common infections. Over the last 25 years, the amount of new drugs entering the clinic has decreased yet bacteria are continually developing resistance to most of the available drugs, making the situation critical (Silver, 2011).

Nowadays, most of the antimicrobials found in the market are either from natural Fproduct, semi synthetic or synthetic (Lahlou, 2013) that are administered orally, parenterally or topically (Phillips *et al.*, 2004). Nevertheless, natural antimicrobials are gaining more attention due to the concern on chemical preservatives in synthetic antimicrobials although chemical preservatives are approved for human intake. Synthetic antimicrobial contain several undesired toxic effects including carcinogenic and teratogenic toxicity especially in the food industry, and it generally has low degradation in the environment (Hayek *et al.*, 2013, Lahlou, 2013). Demonstrated by fluoroquinolones, a class of synthetic antimicrobial agents affected the ecology of intestine microflora and overgrowth of *Candida* was observed (Sullivan *et al.*, 2001). Together with the side effects of synthetic antimicrobials, challenge of AMR pathogens and the emergence of new pathogens demand for the new drugs from natural sources.

2.4.3 Cancer

Cancer is one of the NCDs characterized by uncontrolled growth and spread of abnormal cells. This disease can affect the human immune systems by a series of an event; spreading the abnormal cells, destroys tissues and eventually result in death (National Institutes of Health (US), 2015). Cancer can be caused by both external and internal factors; either they act together or in sequence. Examples of external factors include excessive use of tobacco, unhealthy diet intake and effects of infectious diseases, meanwhile, internal factors such as inherited genetic mutations, immune conditions, and hormones (Cancer Facts and Figure, 2015).

Cancer is a worldwide problem and the leading cause of morbidity and mortality with approximately 14.1 million new cases and 8.2 million cancer-related-deaths in 2012 (Stewart and Wild, 2014), with 2.9 million in developed countries and 5.3 million in developing countries (International Agency for Research on Cancer (IARC), 2012). According to IARC, by year 2030, the number of new cases is expected to increase up to 21.7 million and 13 million cancer deaths.

Efforts to treat the different types of cancer have not achieved the expected results as the mortality rates keep increasing since the last few years (Lambert *et al.,* 2011). Some conventional cancer treatment such as chemotherapy and radiotherapy still remain as an efficient and important treatment. Another treatment is known as targeted cancer therapies or sometimes called molecularly targeted drugs, molecularly targeted therapies or precision medicines (National Cancer Institute, 2016). However, the major challenge in cancer treatment is due to the limitation, side effects and resistance towards the treatment and therapies. Moreover, these treatments are dependent on the type of cancer and the stage of tumor development in the body.

The cytotoxic agents used in treatment are grouped based on their chemical structure and mechanism of actions, affect by damaging and weakening the immune system. In several cases, these treatments might increase the risks of developing different types of cancer future in life (Pardee *et al.*, 2008).

The search for a better cytotoxic agent continues to be an important effort in the discovery of modern anticancer drugs (Gordaliza, 2007). The current research on anticancer drugs is focused on the development of new and more effective chemotherapeutic agents with low to no toxicity to the patients. One of the focuses has been centered on natural products from biological sources such as microorganism.

2.4.4 Neurodegenerative diseases

Neurodegenerative diseases are incurable, debilitating conditions; they result in progressive degeneration or death of nerve cells and have a strong link to age. They include Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD), Huntington's disease, Amyotrophic Lateral Sclerosis (ALS), multiple sclerosis and Creutzfeldt-Jakob disease. A common feature of neurodegenerative diseases is an extended time course until sufficient abnormal protein accumulates, followed by a cascade of symptoms over many years with increasing disability (Jellinger, 2009). Neurodegenerative diseases continuously cause huge economic and social impacts in family members and on healthcare systems in many countries. Report from WHO showed that brain disorders emerge as a leading contributor to global disease burden, estimated based on Disability Adjusted Life Years (DALYs).

The demand of the new drugs is not only important to infectious diseases, but also as therapeutic agents in neurodegenerative diseases. Ideally, treatment would return the cell to a fully functioning state, though pragmatically, lowering damage or stopping total cell destruction is the only realistic outcome. Unfortunately, although many compounds have made it to clinical trials most fail to reach the market due to unfavorable toxicity, or lack of efficiency *in vivo* (Cheng *et al.*,2004; Palmer, 2011). Nowadays, many synthetic compounds studied were not specifically considered as neuroprotective agents but were accidentally found to have activities during repetitive screening programs for drug discovery. Alternatively, an ideal reservoir for the discovery of neuroprotective compounds exists in biologically active natural products, either bacterial, marine extracts or plant, or endogenous peptides and proteins (Levi and Brimble, 2004).

2.5 Rare Actinobacteria – alternative sources of drug discovery

Recently, rare Actinobacteria is proven to produce some unique source of novel bioactive compounds. The rare Actinobacteria strain from different genera namely *Actinomadura, Dactylosporangium, Kibdelospoangium, Amycolatopsis, Saccharopolyspora, Actinoplanes, Micromonospora, Nocardia, Streptoverticillium* and *Streptosporangium* have been proven as major sources of commercial biomolecules (Solanki *et al.*, 2008; Subramani and Aalbersberg, 2012; Jose and Jebakumar, 2013). The list has been further been extended by recent report of bioactive compounds from other genus such as *Nonomuraea* (Beltrametti *et al.*, 2003; Flatt *et al.*, 2013), *Pseudonocardia* (Oh *et al.*, 2009; Carr *et al.*, 2012), *Saccharothrix* (Mukarami *et al.*, 2009; Nakae *et al.*, 2013) and *Actinosynnema* (Siyu-Mao *et al.*, 2012). Uniquely, rare

Actinobacteria are able to produce unprecedented and sometimes complicated bioactive compounds possessing good bioactive potency with low toxicity (Kurtböke, 2012). An example, abbyssomicins, a potent polycyclic polyketides produced by the *Verrucosispora maris* exhibited activity against methicillin-resistant *Staphylococcus aureus* (Riedlinger *et al.*, 2004). Pettit *et al.* (2007) showed that a new bioactive compound known as kitastatin 1 which was isolated from Alaskan *Kitasatospora* sp. exhibited anticancer activity. Table 2.13 lists other examples of bioactive compounds produced by different rare Actinobacteria from various sources.

Table 2.13: Bioactive compound(s) produced by rare Actinobacteria.

Producing species	Bioactive compound(s)	References
Antibacterial agents		
Verrucosispora maris	Abyssomycin C	Riedlinger *et al.* (2004)
Micromonospora	Anthracyclin	Grein *et al.* (1980)
Micromonospora	Clostomicins	Omura *et al.* (1986)
Micromonospora purpurea	Gentamicin	Weinstein *et al.* (1963)
Marinispora	Marinomycin	Kwon *et al.* (2006)
Micromonospora	Netamicin	Berdy (2005)
Nocardia lurida	Ristocetin,	William *et al.* (1980)
Nocardia lurida	Benzanthrins A and B	Theriault *et al.* (1986)
Micromonospora	Thiocoraline	Perez *et al.* (1997)
Amycolatopsis orientalis	Vancomycin	Brigham and Pittenger (1956)
Saccharopolyspora erythraea	Erythromycin	Mc Guire *et al.* (1952)
Amycolatopsis sp. MI481-42F4	Amythiamicins	Shimanaka *et al.* (1994)
Amycolatopsis mediterranei U-32	Rifamycin	Zhang *et al.* (2000)
Pseudonocardia azurea	Azureomycins A and B	Omura *et al.* (1979)
Janibacter limosus	Helquinoline	Asolkar *et al.* (2004)

	Antifungal agents	
Nocardia transvalensis	Transvalencin	Hoshino *et al.* (2004)
	Antiviral agents	
Actinomadura pelletieri	MM461156	Ashton *et al.* (1990)
	Anticancer agents	
Salinispora arenicola	Saliniketals	William *et al.* (2007b)
Kitasatospora	Kitastatin 1	Pettit *et al.* (2007)
Marinispora	Marinomycin	Kwon *et al.* (2006)
Salinispora tropica	Salinosporamide A	William *et al.* (2005)
Salinispora pacifica CNS-237	Salinipyrones, Pacificanonces	Oh *et al.* (2008)
	Cytotoxic agents	
Nocardiopsis lucentensis	Lucentamycins	Cho *et al.* (2007)
Mechercharimyces asporophorigenes	Urukthapelstatin	Matsuo *et al.* (2007)
Salinispora arenicola	Arenamides	Asolkar *et al.* (2008)
Streptoverticillium luteoverticillatum	Butenolides	Li *et al.* (2006)
Micromonospora neihuensis	Neihumicin	Yokoi *et al.* (1988)
	Antitumor agents	
Micromonospora	Anthraquinones	Igarashi *et al.* (2007)
Micromonospora	Thiocoraline	Romero *et al.* (1997)
Micromonospora	Diazepinomicin	Romila *et al* (2004)
Micromonospora	Lomaiviticins	He *et al.* (2001)
Micromonospora	Lupinacidins	Igarashi *et al.* (2011)
Thermoactinomyces	Mechercharmycin	Kanoh *et al.* (2005)
Micromonospora	Arisostatins A and B	Furumai *et al.* (2000)
Salinispora arenicola	Arenicolides	William *et al.* (2007a)
Salinispora chartreusis	Chartreusin	Xu *et al.* (2005)
	Antifungal agents	
Nocardia transvalensis	Transvalencin	Hoshino *et al.* (2004)
	Antiviral agents	

Actinomadura pelletieri	MM461156	Ashton *et al.* (1990)
Immunosuppressive agent		
Nocardia brasiliensis	Brasilicardin	Komatsu *et al.* (2004)
Immunostimulatory agents		
Nocardia rubra	Rubratin	de Reijke *et al.* (1997)

Microorganisms such as Actinobacteria have developed exceptional metabolic and physiological abilities to be able to survive in extreme environments that led them to produce different types of bioactive compounds. Moreover, some of the bioactive compounds are uniquely produced only by certain species. Extensive screening and discovery on novel rare Actinobacteria especially from mangrove has proved they are also prolific sources of novel and various bioactive compounds. In the present study, novel rare Actinobacteria are isolated from mangrove environments and screened for its ability to produce bioactive compounds with activities such as antibacterial, cytotoxic and neuroprotective agents.

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Chapter 3

Microbacterium mangrovi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil

3.1 Declaration for Chapter 3

Monash University

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and conduct, data analysis, manuscript preparation	55

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Lee Learn Han	Experimental design, result discussion, data analysis, manuscript preparation	NA
Nurullhudda Zainal	Participated in experiment	NA
Eng Shu Kee	Participated in experiment	5
Nurul Syakima Ab. Mutalib	Result discussion	NA
Yin Wai Fong	Result discussion	NA
Chan Kok Gan	Experimental design, result discussion, data analysis, manuscript preparation	NA

*NA – not a student registered under Monash University

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12th May 2016
Main Supervisor's Signature	[redacted]	**Date** 3rd May 2016

3.2 Summary of Chapter 3

The genus of *Microbacterium* was proposed by Orla-Jensen (1919) for Gram-positive, rod shape, non-spore forming bacteria that were isolated from the studies on lactic acid producing bacteria (Kim *et al.*, 2008). *Microbacterium* has been isolated from diverse habitat such as soils (Anand *et al.*, 2012), plants (Madhaiyan *et al.*, 2010; Young *et al.*, 2010), water (Kim *et al.*, 2008), dairy product (Brennan *et al.*, 2001), cosmetic products (Bakir *et al.*, 2008) and human (Clermont *et al.*, 2009). The description of the strains has been amended by Collin *et al.* (1983) and more recently by Takeuchi and Hatano (1998). The finals amendment concluded that the genus *Aureobacterium* was united with the genus *Microbacterium* (Krishanmurthi *et al.*, 2012). Althought the diamino acid in the cell wall of these two genera differed, the two genera are intermixed phylogenetically and share similar chemotaxonomic and physiological characterization (Takeuchi and Hatano, 1998). At the time of writing (March, 2016), there is 96 species with validly published names of the genus *Microbacterium* (http://www.bacterio.net/microbacterium.html).

The objective of this study was to determine the taxonomic status of strain MUSC 115^T using polyphasic approach on the basis of chemotaxonomic, molecular and phenotypic analysis; which showed that strain MUSC 115^T is a different and novel species under the genus of *Microbacterium.*

Results from polyphasic approach including genotypic, chemotaxonomic and phenotypic characterization proved that strain MUSC 115^T was different from all other species in the genus

of *Microbacterium*. Thus, strain MUSC 115^{T} was proposed to be classified as new species named *Microbacterium mangrovi* sp. nov.

International Journal of Systematic and Evolutionary Microbiology (2014), **64**, 3513–3519 DOI 10.1099/ijs.0.062414-0

Microbacterium mangrovi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil

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Strain MUSC 115^T was isolated from mangrove soil of the Tanjung Lumpur river in the state of Pahang, Peninsular Malaysia. Cells of this strain stained Gram-positive and were non-spore-forming, short rods that formed yellowish-white colonies on different agar media. The taxonomy of strain MUSC 115^T was studied by a polyphasic approach, and the organism showed a range of phylogenetic and chemotaxonomic properties consistent with those of the genus *Microbacterium*. The cell-wall peptidoglycan was of type B2β, containing the amino acids ornithine, alanine, glycine, glutamic acid and homoserine. The muramic acid was of the *N*-glycolyl form. The predominant menaquinones detected were MK-12, MK-13 and MK-11. The polar lipids consisted of phosphatidylglycerol, phosphoglycolipid, diphosphatidylglycerol, two unidentified lipids, three unidentified phospholipids and four unidentified glycolipids. The major fatty acids of the cell membrane were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The whole-cell sugars detected were ribose, glucose, mannose and galactose. Based on the 16S rRNA gene sequence, strain MUSC 115^T showed the highest sequence similarity to *Microbacterium immunditiarum* SK 18^T (98.1 %), *M. ulmi* XIL02^T (97.8 %) and *M. arborescens* DSM 20754^T (97.5 %) and lower sequence similarity to strains of other species of the genus *Microbacterium*. DNA–DNA hybridization experiments revealed a low level of DNA–DNA relatedness (less than 24 %) between strain MUSC 115^T and the type strains of closely related species. Furthermore, BOX-PCR fingerprint comparison also indicated that strain MUSC 115^T represented a unique DNA profile. The DNA G+C content determined was 70.9 ± 0.7 mol%, which is lower than that of *M. immunditiarum* SK 18^T. Based on the combination of genotypic and phenotypic data, it is proposed that strain MUSC 115^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium mangrovi* sp. nov. is proposed. The type strain is MUSC 115^T (=MCCC 1K00251^T=DSM 28240^T=NBRC 110089^T).

Members of the genus *Microbacterium*, established by Orla-Jensen (1919), the description of which has been emended by Collins *et al.* (1983), Takeuchi & Hatano (1998) and Krishnamurthi *et al.* (2012), are widespread in nature and have been isolated from diverse habitats such as soil, plants, water, milk products, insects and humans (Collins & Bradbury, 1992; Richert *et al.*, 2007; Dastager *et al.*, 2008; Madhaiyan *et al.*, 2010; Krishnamurthi *et al.*, 2012). At the time of writing, the genus *Microbacterium* contained 84 species with validly published names (http://www.bacterio.net/microbacterium.html). According to Schumann *et al.* (1999), DNA–DNA hybridization assays are vital to the identification of novel species of the genus *Microbacterium*, since the 16S rRNA gene sequence similarity between the closest related type strains is more than 97 % in some

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MUSC 115^T is KF028598.

Three supplementary figures and a supplementary table are available with the online Supplementary Material.

strains and 98–99 % in many strains and can be as high as 99.9 %, as described for the type strains of *Microbacterium oxydans* and *Microbacterium luteolum* (Schumann *et al.*, 1999). Based on the data from a polyphasic approach, we here describe a novel species of the genus *Microbacterium*, *Microbacterium mangrovi* sp. nov., for a bacterium isolated from a mangrove environment in Malaysia.

A study was undertaken to investigate the bacterial diversity in a mangrove forest with the aim of isolating amylolytic strains. A series of bacteria were isolated from mangrove soil samples, one of which produced significant starch hydrolysis activity and was chosen for further study. Polyphasic analysis of this strain, MUSC 115^T, suggested that it belonged to the genus *Microbacterium* (Takeuchi & Hatano, 1998). Strain MUSC 115^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located in the mangrove forest of Tanjung Lumpur in the state of Pahang, Peninsular Malaysia, in December 2012. Topsoil samples of the upper 20 cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored in −20 °C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C; Takahashi *et al.*, 1996). Five grams of the pretreated air-dried soil was mixed with 45 ml sterilized water and mill-ground, spread onto a selective isolation medium, starch casein agar (SCA; Küster & Williams, 1964) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and then incubated at 28 °C for 7 days. Pure cultures of strain MUSC 115^T were maintained on ISP2 medium at 28 °C and as glycerol suspensions (20 %, v/v) at −20 °C.

Cultural characteristics of strain MUSC 115^T were determined following growth on ISP2 and ISP7 media (Shirling & Gottlieb, 1966), SCA (Küster & Williams 1964), *Streptomyces* agar (SA; Atlas 1993), actinomycetes isolation agar (AIA; Atlas 1993) and nutrient agar (NA; MacFaddin, 2000) for 7 days at 28 °C. Light microscopy (80*i*; Nikon) and scanning electron microscopy (JEOL-JSM 6400) were used to observe the morphology of the strain after incubation on ISP2 medium at 28 °C for 7 days. The ISCC-NBS colour charts (Kelly, 1964) were used to determine the names and designations of colony colours. Gram staining was performed by the standard Gram reaction and was confirmed by using KOH lysis (Cerny, 1978). Growth was tested at 4–52 °C at intervals of 4 °C on ISP2 medium. The pH range for growth was tested between pH 4.0 and 10.0 at intervals of 1 pH unit. NaCl tolerance was tested using tryptic soy broth (TSB) and salt concentrations ranging from 0 to 18 % (w/v) at intervals of 2 %. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Production of melanoid pigments was examined using tyrosine agar (ISP7 medium). Haemolytic activity was assessed in blood agar medium containing 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) human blood (Carrillo *et al.*, 1996). Plates were examined for haemolysis after incubation at 32 °C for 7 days. Lipase, amylase, cellulase, chitinase, protease and xylanase activities were determined by growing cells on ISP2 medium following the protocols described by Meena *et al.* (2013). The presence of a clear zone around colonies signifies the potential of isolates for surfactant production. Antibiotic susceptibility tests were performed by the disc diffusion method as described by Shieh *et al.* (2003). *Microbacterium immunditiarum* JCM 14034^T, *M. ulmi* JCM 14282^T and *M. arborescens* DSM 20754^T were obtained from the JCM and DSMZ as indicated and tested concurrently with strain MUSC 115^T. Antimicrobials used and the amounts per disc (Oxoid) were as follows: ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), nalidixic acid (30 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). Cells were resistant to nalidixic acid and sensitive to other antimicrobials tested. Carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions. Differential physiological characteristics between strain MUSC 115^T and the type strains of closely related species of the genus *Microbacterium* are described in the species description and in Table 1.

Genomic DNA extraction for PCR was performed as described by Hong *et al.* (2009). The 16S rRNA gene was amplified using the universal primers p27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′). PCR amplification and purification of the product were completed as described by Hong *et al.* (2009). The 16S rRNA gene sequence of strain MUSC 115^T was aligned with sequences of closely related type strains that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL_X software (Thompson *et al.*, 1997). The alignment was verified manually and adjusted prior to the reconstruction of the phylogenetic tree using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms with the MEGA version 5.2 software (Tamura *et al.*, 2011). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) was used for calculations of sequence similarity. The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980).

The nearly complete 16S rRNA gene sequence was established for strain MUSC 115^T (1484 bp) and phylogenetic trees were reconstructed to determine the phylogenetic position of this strain (Fig. 1 and Fig. S1, available in the online Supplementary Material). Phylogenetic analysis exhibited that strain MUSC 115^T was closely related to *M. immunditiarum* SK 18^T, as they formed a distinct clade supported by a high bootstrap value (86 %), indicating a high confidence level of this association

Table 1. Differentiation of strain MUSC 115^T and the type strains of closely related species of the genus *Microbacterium*

Strains: 1, MUSC 115^T; 2, *M. immunditiarum* JCM 14034^T; 3, *M. ulmi* JCM 14282^T; 4, *M. arborescens* DSM 20754^T. Data were obtained in this study. +, Positive; −, negative; (+), weakly positive. All strains were positive for growth in 1 % NaCl and utilization of dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, lactose, melibiose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, 3-methyl glucose, inosine, D-sorbitol, D-mannitol, glycerol, gelatin, glycyl L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-serine, pectin, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, *p*-hydroxyphenylacetic acid, D-lactic acid methyl ester, L-lactic acid, L-malic acid, Tween 40, γ-aminobutyric acid, α-hydroxybutyric acid, α-ketobutyric acid, acetoacetic acid, propionic acid and acetic acid.

Characteristic	1	2	3	4
Major fatty acids (%)				
anteiso-$C_{15:0}$	38.0	22.0	35.1	42.9
anteiso-$C_{17:0}$	31.2	39.2	30.0	24.9
iso-$C_{16:0}$	8.6	19.2	16.7	28.5
Colony colour on:*				
ISP2 agar	YWH	CY	WH	DO
SA	YWH	POY	PWH	MRO
NA	YWH	PYWH	PWH	MOY
Luria–Bertani agar	YWH	PYWH	PWH	MOY
Growth at/in:				
16 °C	+	+[a]†	+[b]	+
32 °C	+	+[a]	+[b]	−[c]
40 °C	(+)	(+)	−[b]	−[c]
pH 5	+	−[a]	+[b]	−
pH 9	+	−[a]	−[b]	−
4 % NaCl	+	−	+	+
8 % NaCl	−	−	+[b]	+
Catalase	+	+[a]	−[b]	+[c]
Haemolytic activity	−	−	−	+
Hydrolysis of:				
Starch (amylase)	+	−	+[b]	−[c]
CM-cellulose	−	+	+[b]	+
Casein	−	−[a]	+[b]	−
Xylan	−	−[a]	+[b]	−
Carbon source utilization				
Stachyose	+	−	+	+
Raffinose	+	−	+	+
N-Acetyl-β-D-mannosamine	+	−	+	+
N-Acetyl-D-galactosamine	+	−	+	−
D-Galactose	−	+	+	+
D-Fucose	−	+	+	+
L-Fucose	−	+	+	+
L-Rhamnose	−	+	+	+
D-Arabitol	+	−	+	+
myo-Inositol	+	−	+	+
D-Glucose 6-phosphate	−	+	+	+
D-Fructose 6-phosphate	−	+	+	+
D-Aspartic acid	+	−	+	−
D-Serine	−	+	+	−
L-Pyroglutamic acid	+	−	+	+
Glucuronamide	−	+	+	+
Mucic acid	+	−	+	−
Quinic acid	+	−	+	−
D-Saccharic acid	+	−	+	−
Methyl pyruvate	+	−	+	+
α-Ketoglutaric acid	−	+	+	+
D-Malic acid	+	−	+	+
Formic acid	+	−	+	−
Chemical sensitivity assays				
Troleandomycin	−	+	+	+
Lincomycin	−	+	+	+
Tetrazolium violet	−	+	+	+
Tetrazolium blue	−	+	+	+
Lithium chloride	+	−	+	+
Sodium butyrate	+	−	+	+
Sodium bromate	−	+	+	+

*CY, Creamish yellow; DO, dirty orange; MOY, moderate orange–yellow; MRO, moderate reddish orange; POY, pale orange–yellow; PWH, pale white; PYWH, pale yellowish white; WH, white; YWH, yellowish white.

†Result in accordance with that published for: *a*, *M. immunditiarum* SK 18^T by Krishnamurthi *et al.* (2012); *b*, *M. ulmi* XIL02^T by Rivas *et al.* (2004); *c*, *M. arborescens* DSM 20754^T by Takeuchi & Hatano (1998).

(Fig. 1). This association was also supported in the phylogenetic tree reconstructed using the maximum-likelihood algorithm (Fig. S1). 16S rRNA gene sequence analysis of strain MUSC 115^T showed the highest similarity to *M. immunditiarum* SK 18^T (98.1 %), followed by *M. ulmi* XIL02^T (97.8 %) and *M. arborescens* DSM 20754^T (97.5 %); sequences similarities of less than 97.5 % were obtained to the type strains of other species of the genus *Microbacterium*. According to Stackebrandt & Goebel (1994), a 16S rRNA gene sequence divergence of more than 3 % is generally accepted as a criterion for delineating different species. The sequence divergence between MUSC 115^T and the most closely related type strain, *M. immunditiarum* SK 18^T, was 1.9 %, a value that indicated a strong possibility of strain MUSC 115^T representing a novel species of the genus *Microbacterium*.

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies were obtained after growth in TSB at 28 °C for 7 days on a rotary shaker. DNA–DNA hybridization analysis of strain MUSC 115^T and closely related type strains was performed by the Identification Service of the DSMZ. Extraction of genomic DNA from strain MUSC 115^T, *M. immunditiarum* JCM 14034^T, *M. ulmi* JCM 14282^T and *M. arborescens* DSM 20754^T was performed as described by Cashion *et al.* (1977). DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a

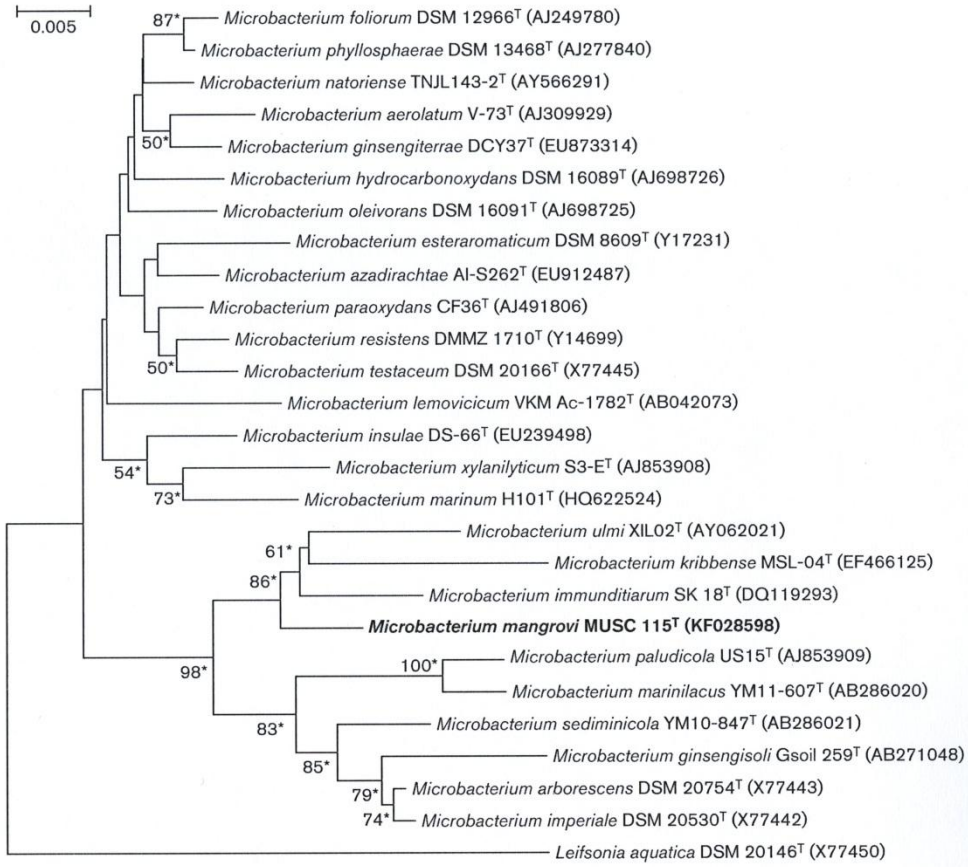

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on 16S rRNA gene sequences showing relationships between strain MUSC115^T and representatives of related taxa. Bootstrap values (>50 %) based on 1000 resampled datasets are shown at branch nodes. Bar, 5 substitutions per 1000 nucleotide positions. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood algorithm. *Leifsonia aquatica* DSM 20146^T was used as an outgroup.

model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). The DNA–DNA relatedness between strain MUSC 115^T and *M. immunditiarum* JCM 14034^T (23.6 ± 0.5 %), *M. ulmi* JCM 14282^T (26.2 ± 2.7 %) and *M. arborescens* DSM 20754^T (16.3 ± 1.1 %) was significantly lower than 70 %, the recommended threshold value for the delineation of genomic species (Wayne *et al.* 1987). These results suggested that strain MUSC 115^T did not belong to any of these species. Moreover BOX-PCR fingerprint analysis was used to compare strain MUSC 115^T and the reference strains using the primer BOX-A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic *et al.*, 1991). BOX-PCR cycling parameters were 5 min at 94 °C for pre-denaturation, 35 cycles each of 30 s at 94 °C for denaturation, 30 s at 53 °C for annealing and 7 min at 65 °C for extension and a final extension at 65 °C for 8 min. The PCR products were visualized by 2 % agarose gel electrophoresis. The rep-PCR results are shown in Fig. S2. It is evident that strain MUSC 115^T yielded a unique BOX-PCR fingerprint compared with closely related type strains. These results are in agreement with the results of DNA–DNA hybridization, that strain MUSC 115^T represents a novel species.

Cellular fatty acid analysis of strain MUSC 115^T and closely related type strains was carried out by the Identification Service of the DSMZ. Cell mass of strain MUSC 115^T and the reference strains was harvested from TSB after incubation at 28 °C for 5 days. The fatty acids were extracted and prepared according to the standard protocol of the MIDI System (Sasser, 1990). The complete fatty acid profiles of strain MUSC 115^T and the reference strains are

shown in Table S1. The predominant cellular fatty acids in strain MUSC 115^T were anteiso-$C_{15:0}$ (38.0 %) and anteiso-$C_{17:0}$ (31.2 %). Other fatty acids detected were iso-$C_{16:0}$ (8.6 %), iso-$C_{18:0}$ (6.7 %), iso-$C_{17:0}$ (5.6 %) and trace amounts of $C_{18:0}$, iso-$C_{15:0}$ and anteiso-$C_{19:0}$ (Table S1). The fatty acid profile of strain MUSC 115^T was similar to those of the reference strains, which also contained the predominant fatty acids anteiso-$C_{15:0}$ (22.0–42.9 %) and anteiso-$C_{17:0}$ (24.9–39.2 %) (Table S1). Strain MUSC 115^T contained a fatty acid profile that was quantitatively different from that of *M. immunditiarum* JCM 14034^T, particularly in the amounts of anteiso-$C_{15:0}$ and iso-$C_{16:0}$ (Tables 1 and S1). These fatty acids were present in almost equal amounts (22.0 and 19.2 %) in *M. immunditiarum* JCM 14034^T whereas, for strain MUSC 115^T, the amount of anteiso-$C_{15:0}$ (38.0 %) was nearly double that found in *M. immunditiarum* JCM 14034^T.

Analysis of the peptidoglycan amino-acid composition and whole-cell sugars of strain MUSC 115^T was carried out by Identification Service of the DSMZ. The analyses were carried out according to Schumann (2011). The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan of strain MUSC 115^T contained the amino acids ornithine (Orn), alanine, glycine, glutamic acid and homoserine (Hse). The molar ratio was 1.0 Orn : 0.7 Hse : 1.4 Gly : 0.5 Ala : 1.0 Glu. 3-*threo*-hydroxyglutamic acid could not be detected. The identity of these amino acids was confirmed by agreement in the GC retention time with those of authentic standards and by the detection of characteristic MS fragment ions of their derivatives. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C) of the peptidoglycan contained the peptides Orn–D-Ala, Gly–Orn and Gly–Glu. From these analytical data, it was concluded that strain MUSC 115^T contained the peptidoglycan type B2β (Schleifer & Kandler, 1972). The *N*-acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida & Aida (1977). The hydrolysate (0.5 M H_2SO_4, 2 h, 100 °C) of cells of strain MUSC 115^T contained ribose, glucose, mannose and galactose. Galactose has been detected in many other species of the genus *Microbacterium* (Rivas *et al.*, 2004; Krishnamurthi *et al.*, 2012). The detection of ribose, glucose and mannose was noteworthy, as these sugars were not found in the closely related type strains *M. immunditiarum* JCM 14034^T and *M. ulmi* JCM 14282^T, exhibiting a significant difference between strain MUSC 115^T and these type strains.

Analysis of respiratory quinones and polar lipids was carried out by the Identification Service of the DSMZ. The cellular polar lipids were extracted and analysed by TLC (Kates, 1986). The predominant menaquinones detected were MK-12 (62 %), MK-13 (28 %) and MK-11 (8 %). A minor amount of MK-10 (1 %) was also detected. The polar lipids consisted of phosphatidylglycerol, phosphoglycolipid, diphosphatidylglycerol, two unidentified lipids, three unidentified phospholipids and four unidentified glycolipids (Fig. S3a). Comparison of the polar lipid profiles of strain MUSC 115^T and the closest related type strain *M. immunditiarum* JCM 14034^T (Fig. S3b) indicated that they shared four types of polar lipid, namely unidentified lipids and glycolipids, phosphatidylglycerol and diphosphatidylglycerol. However, differences in the polar lipid profiles, e.g. strain MUSC 115^T contained phospholipid and phosphoglycolipid, which were lacking in *M. immunditiarum* JCM 14034^T, indicated that strain MUSC 115^T was distinct.

Genomic DNA for the determination of G+C content was extracted according to Cashion *et al.* (1977) and the G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). The DNA G+C content of strain MUSC 115^T was 70.9±0.7 mol%, within the range of 69.0–76.6 mol% described for species within the genus *Microbacterium* (Takeuchi & Hatano, 1998; Rivas *et al.*, 2004; Krishnamurthi *et al.*, 2012). On the basis of phenotypic, chemotaxonomic, DNA–DNA hybridization and phylogenetic analyses, strain MUSC 115^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium mangrovi* sp. nov. is proposed.

Description of *Microbacterium mangrovi* sp. nov.

Microbacterium mangrovi (man.gro'vi. N.L. gen. n. *mangrovi* of a mangrove).

Cells stain Gram-positive and are non-spore-forming, irregular cocci or rods. Cells form yellowish-white colonies on all agar media tested. Good growth is observed on ISP2 medium, NA and SA after 7 days at 28 °C; cells grow moderately well on Luria–Bertani agar, whereas cells grow poorly on SCA, ISP7 medium and AIA. Using TSB, the ranges of NaCl concentration, temperature and pH for growth are 0–4 % (w/v) NaCl, 15–36 °C and pH 5.0–8.0; optimal growth occurs at 0–2 % NaCl, 28–32 °C and pH 5.0–8.0. Cells are positive for catalase but negative for haemolytic activity. Soluble starch is hydrolysed, but CM-cellulose, chitin, tributyrin (lipase), casein and xylan are not. The following compounds are utilized as sole carbon sources: dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, lactose, melibiose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, α-D-glucose, D-mannose, D-fructose, 3-methyl glucose, inosine, D-sorbitol, D-mannitol, D-arabitol, *myo*-inositol, glycerol, D-aspartic acid, gelatin, glycyl L-proline, pectin, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxyphenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, Tween 40, γ-aminobutyric acid, α-hydroxybutyric acid, β-hydroxy-DL-butyric acid, α-ketobutyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid. The following compounds are not utilized as sole carbon sources: D-galactose, D- and L-fucose, L-rhamnose, D-glucose 6-phosphate, D-fructose 6-phosphate, D-serine, L-galactonic acid lactone, glucuronamide, citric acid, α-ketoglutaric acid and bromosuccinic acid. L-Alanine, L-arginine, L-aspartic acid, L-glutamic acid,

L-histidine, L-pyroglutamic acid and L-serine are utilized as sole nitrogen sources. In chemical sensitivity assays, cells are sensitive towards fusidic acid, D-serine, troleandomycin, minocycline, lincomycin, guanine hydrochloride, Niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue and sodium bromate, while cells are resistant to 1 % sodium lactate, rifamycin RV, nalidixic acid, lithium chloride, potassium tellurite, aztreonam and sodium butyrate. Cells are sensitive to (per disc) ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). Resistant to nalidixic acid (30 μg). The peptidoglycan is of type B2β, containing the amino acids ornithine, alanine, glycine, glutamic acid and homoserine. The acyl type is *N*-glycolyl. The predominant menaquinones are MK-12, MK-13 and MK-11. The major fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The whole-cell sugars are ribose, glucose, mannose and galactose. The polar lipids consist of phosphatidylglycerol, phosphoglycolipid, diphosphatidylglycerol, two unidentified lipids, three unidentified phospholipids and four unidentified glycolipids.

The type strain is MUSC 115^T (=MCCC 1K00251^T=DSM 28240^T=NBRC 110089^T), isolated from mangrove soil collected from the Tanjung Lumpur mangrove forest in the state of Pahang, Peninsular Malaysia. The G+C content of the genomic DNA of the type strain is 70.9±0.7 mol%.

Acknowledgements

This work was supported by a University of Malaya for High Impact Research grant (UM-MOHE HIR Nature Microbiome grant no. H-50001-A000027) awarded to C. K.-G and an External Industry Grant (vote no. GBA-808138) awarded to L. L.-H. The authors are grateful to researchers from the DSMZ for performing the chemotaxonomic analyses and to Professor Jean Euzéby for support in providing the Latin etymology of the new name.

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Chapter 4

Sinomonas humi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil

4.1 Declaration for Chapter 4

Monash University

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and conduct, data analysis, manuscript preparation	55

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Lee Learn Han	Experimental design, result discussion, data analysis, manuscript preparation	NA
Nurullhudda Zainal	Participated in experiment	NA
Nurul Syakima Ab. Mutalib	Result discussion	NA
Yin Wai Fong	Result discussion	NA
Chan Kok Gan	Experimental design, result discussion, data analysis, manuscript preparation	NA

*NA – not a student registered under Monash University

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12th May 2016
Main Supervisor's Signature	[redacted]	**Date** 3rd May 2016

4.2 Summary of Chapter 4

The genus of *Sinomonas* was proposed by Zhou *et al.* (2009) with the description of newly isolated strain *Sinomonas flava* CW 108^T and the reclassification of *Arthrobacter atrocyaneus* as *Sinomonas atrocyanea* (type strain DSM 20127^T) (Zhou *et al.*, 2012). *Sinomonas* means a bacterium isolated from China and the strain included members that were isolated from different source and environment. At the time of writing this report (March, 2016), the genus of *Sinomonas* consisted of 8 species with validly published name (http://www.bacterio.net/sinomonas.html). In this study, it is evident that the genus of *Sinomonas* is not commonly found in the mangrove environment since this genus was mostly discovered in forest soils (Zhou *et al.*, 2009), polluted forest soil (Zhou *et al.*, 2012), volcanic rocks (Zhou *et al.*, 2012), rhizosphere soil (Zhang *et al.*, 2015) and the surface of weathered biotite (Bao *et al.*, 2015). Therefore, this strain represents the first *Sinomonas* species from mangrove.

The objective of this study was to determine the taxonomic status of strain MUSC 117^T using polyphasic approach on the basis of genotypic, chemotaxonomic and phenotypic analysis and to show that strain MUSC 117^T is different and novel species under the genus of *Sinomonas.*

Results from polyphasic approach including genotypic, chemotaxonomic and phenotypic characterization proved that strain MUSC 117^T is different from all other species in the genus of *Sinomonas*. Thus, strain MUSC 117^T is proposed to be classified as new species named *Sinomonas humi* sp. nov.

International Journal of Systematic and Evolutionary Microbiology (2015), **65**, 996–1002 DOI 10.1099/ijs.0.000053

Sinomonas humi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil

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Strain MUSC 117^T was isolated from mangrove soil of the Tanjung Lumpur forest in Pahang, Malaysia. This bacterium was yellowish-white pigmented, Gram-staining-positive, rod–coccus shaped and non-motile. On the basis of 16S rRNA gene sequence, strain MUSC 117^T exhibited highest sequence similarity to *Sinomonas atrocyanea* DSM 20127^T (98.0 %), *Sinomonas albida* LC13^T (97.9 %) and *Sinomonas soli* CW 59^T (97.8 %), and lower (<97.6 %) sequence similarity to other species of the genus *Sinomonas*. DNA–DNA hybridization experiments revealed a low level of DNA–DNA relatedness (less than 27 %) between strain MUSC 117^T and closely related species. Chemotaxonomically, the peptidoglycan type was A3α, containing the amino acids lysine, serine, glycine, alanine, glutamic acid and muramic acid. The whole-cell sugars detected were rhamnose, ribose, glucose, galactose and a smaller amount of mannose. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and five unidentified glycolipids. The major fatty acids (>10.0 %) of the cell membrane were anteiso-$C_{15:0}$ (39.4 %), $C_{18:1}\omega7c$ (17.7 %), anteiso-$C_{17:0}$ (17.2 %) and iso-$C_{16:0}$ (11.4 %). The predominant respiratory quinones detected were MK-9(H_2) and MK-9. The DNA G+C content was 67.3 mol%. A comparison of BOX-PCR fingerprints indicated that strain MUSC 117^T represented a unique DNA profile. Results based on a polyphasic approach showed that strain MUSC 117^T represents a novel species of the genus *Sinomonas*, for which the name *Sinomonas humi* sp. nov. is proposed. The type strain of *Sinomonas humi* sp. nov. is MUSC 117^T (=DSM 29362^T=MCCC 1K00410^T=NBRC 110653^T).

The genus *Sinomonas* was established by Zhou *et al.* (2009) with the description of the strain *Sinomonas flava* CW 108^T and the reclassification of *Arthrobacter atrocyaneus* as *Sinomonas atrocyanea* (type strain DSM 20127^T) (Kuhn & Starr, 1960; Zhou *et al.*, 2009). Following the publication of the genus *Sinomonas*, the description of *Sinomonas soli* and reclassification of *Arthrobacter echigonensis* and *Arthrobacter albidus* (Ding *et al.*, 2009) as *Sinomonas echigonensis* and *Sinomonas albida* occurred (Zhou *et al.*, 2012). At the time of writing, the genus *Sinomonas* comprised seven species (http://www.bacterio.net/): the type species *S. flava* and *S. atrocyanea* (Zhou *et al.*, 2009), *S. albida*, *S. echigonensis*, *S. soli*, '*Sinomonas notoginsengisoli*' and '*Sinomonas mesophila*' (Zhou *et al.*, 2012; Prabhu *et al.*, 2014; Zhang *et al.*, 2014). Members of these species were isolated from habitats such as polluted forest soil and filtration substrate (Ding *et al.*, 2009; Zhou *et al.*, 2009, 2012). DNA–DNA hybridization assays were vital for the identification of novel species in the genus *Sinomonas* since the 16S rRNA gene sequence similarity between closely related type strains is more than 98 % for strains *S. albida* LC13^T and *S. echigonensis* LC 10^T, and more than 99.4 % for *S. atrocyanea* DSM 20127^T, *S. flava* CW 108^T and *S. soli* CW 59^T. On the basis of data from a polyphasic approach, we describe a novel species of the genus *Sinomonas*, *Sinomonas humi* sp. nov., an actinobacterial isolate from the mangrove environment in Malaysia.

A study was undertaken to investigate the bacterial diversity in mangrove forest with the aim of isolating

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MUSC 117^T is KC964537.

Two supplementary figures are available with the online Supplementary Material.

amylolytic strains. A series of bacteria were isolated from a mangrove soil sample, one of which produced significant starch hydrolysis activity and was chosen for further study. The polyphasic analysis of this strain, MUSC 117^T, suggested that it belonged to the genus *Sinomonas* (Zhou *et al.*, 2009, 2012). Strain MUSC 117^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located in the mangrove forest of Tanjung Lumpur in the state of Pahang, Peninsular of Malaysia, in December 2012. Topsoil samples of the upper 20 cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored at −20 °C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C; Takahashi *et al.*, 1996). Five grams of the pretreated air-dried soil was mixed with 45 ml sterilized water and mill ground, spread onto isolation medium: starch casein agar (SCA; Küster & Williams, 1964) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), then incubated at 28 °C for 7 days. Pure cultures of strain MUSC 117^T were maintained on ISP2 medium (Shirling & Gottlieb, 1966) at 28 °C and as glycerol suspensions (20 %, v/v) at −20 °C.

Cultural characteristics of strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T, *S. soli* KCTC 19389^T and *S. flava* JCM 16034^T were determined following growth on ISP2 and ISP7 media (Shirling & Gottlieb, 1966), SCA, Streptomyces agar (SA; Atlas, 1993), Actinomycetes isolation agar (AIA; Atlas, 1993) and nutrient agar (MacFaddin, 2000) for 7 days at 28 °C. The Inter-Society Color Council–National Bureau of Standards (ISCC-NBS) colour charts (Kelly, 1964) were used to determine the names and designations of the colony colours. Light microscopy (80*i*; Nikon) and scanning electron microscopy (JSM-6400; JEOL) were used to observe the morphology of the strain after incubation on ISP2 medium at 28 °C for 7 days. Gram staining was performed by using the standard Gram reaction and was confirmed by using KOH lysis (Cerny, 1978). Growth was tested at 10–44 °C at intervals of 4 °C on ISP2 medium. The pH range for growth was tested between pH 4.0 and 10.0 at intervals of 1 pH unit. NaCl tolerance was tested using tryptic soy broth (casein, 17 g; soybean meal, 3 g; glucose, 2.5 g; dipotassium hydrogen phosphate, 2.5 g; distilled water, 1 l; pH 7.3) and NaCl concentrations ranging from 0–18 % (w/v) at intervals of 2 %. Haemolytic activity was assessed in blood agar medium containing 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) human blood (Carrillo *et al.*, 1996). Plates were examined for haemolysis after incubation at 32 °C for 7 days. Amylase, cellulase, chitinase, lipase, protease and xylanase activities were determined by growing cells on ISP2 medium following protocols as described by Meena *et al.* (2013). Presence of a clear zone around colonies signifies the potential of isolates for surfactant production. Catalase activity and production of melanoid pigments were determined following protocols described by Lee *et al.* (2014a). Antibiotic susceptibility tests were performed by using the disc diffusion method as described by Shieh *et al.* (2003). Antimicrobials used and the amounts per disc (Oxoid) were as follows: ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), nalidixic acid (30 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). Carbon source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions. All of the physiological assays mentioned were tested concurrently for strains MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T. These type strains were obtained from NBRC, JCM and KCTC as indicated. Differential physiological characteristics between strain MUSC 117^T and the type strains of closely related species of the genus *Sinomonas* are described in the species description and in Table 1.

Genomic DNA extraction for PCR was performed as described by Hong *et al.* (2009). PCR amplification of the 16S rRNA gene was completed as described by Lee *et al.* (2014b). The 16S rRNA gene sequence of strain MUSC 117^T was aligned with sequences of closely related type strains that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL X software (Thompson *et al.*, 1997). The alignment was verified manually and adjusted prior to the reconstruction of phylogenetic trees using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms with MEGA version 5.2 software (Tamura *et al.*, 2011). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) was used for calculations of sequence similarity. The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980).

The almost complete 16S rRNA gene sequence was established for strain MUSC 117^T (1493 bp) and phylogenetic trees were reconstructed to decide the phylogenetic position of this strain (Figs 1 and 2). Phylogenetic analysis showed that strain MUSC 117^T was closely related to all of the type strains of species of the genus *Sinomonas* as they formed a distinct clade supported by a high bootstrap value (100 %), indicating the highest confidence level of this association (Fig. 1). Moreover strain MUSC 117^T formed a distinctive subclade with type strain *S. albida* LC13^T supported by high bootstrap values of 99 % (Fig. 1) and 97 % (Fig. 2). Analysis of the 16S rRNA gene sequence of strain MUSC 117^T showed that this isolate had highest similarity to the type strain *S. atrocyanea* DSM 20127^T (98.0 %), followed by *S. albida* LC13^T (97.9 %) and *S. soli* CW 59^T (97.8 %), whereas sequence similarities of less than 97.6 % were obtained to other species of the genus *Sinomonas*. DNA–DNA hybridization has been vital in the identification of novel species in the genus *Sinomonas* as the 16S rRNA gene sequence similarities between type

Table 1. Differentiation of strain MUSC 117^T and type strains of closely related species of the genus *Sinomonas*

Strains: 1, *Sinomonas humi* sp. nov. MUSC 117^T; 2, *S. atrocyanea* NBRC 12956^T; 3, *S. albida* JCM 21830^T; 4, *S. soli* KCTC 19389^T; 5, *S. flava* JCM 16034^T. Data from all strains were obtained from this study. +, Positive; −, negative; (+), weakly positive. All strains were amylolytic and positive for growth at 40 °C and assimilation of dextrin, maltose, gentiobiose, turanose, raffinose, α-lactose, melibiose, methyl β-D-glucoside, D-galactose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-arabitol, *myo*-inositol, glycerol, D-fructose 6-phosphate, L-glutamic acid, L-histidine, L-galactonic acid lactone, citric acid, D-malic acid, L-malic acid, γ-aminobutyric acid, α-hydroxybutyric acid, acetic acid and formic acid. All strains were negative for assimilation of D-mannose, L-pyroglutamic and L-serine.

Characteristic	1	2	3	4	5
Colony colour (on AIA)*	YW	YW	YW	POY	PY
Growth at:					
10 °C	(+)	(+)	+†	(+)	(+)
pH 8	+	(+)	(+)†	+‡	+§
4 % NaCl	(+)	+	(+)	−‡	−§
Catalase	+	+‡	+†	+‡	(+)§
Carbon source utilization					
Cellobiose	+	+	+	−	+
Sucrose	+	+	+†	−	+§
Stachyose	+	+	+	−	+
D-Salicin	−	+	+	+	−
α-D-Glucose	+	+	−	−	−
D-Fructose	−	+	−	−	−
D-Fucose	+	−	+	+	+
D-Mannitol	−	−	−	+	+
D-Glucose 6-phosphate	−	+	+	−	+
D-Aspartic acid	+	−	+	+	+
L-Alanine	−	+	−	−	+
Gelatin	−	−	+	−	+
Pectin	−	−	−	+	−
D-Galacturonic acid	−	−	+	+	+
Mucic acid	−	+	+	−	+
α Ketoglutaric acid	+	−	+	+	+
Bromosuccinic acid	−	+	+	−	−
β-Hydroxy-DL-butyric acid	−	+	+	−	+
Acetoacetic acid	−	+	−	−	+
L-lactic acid	−	−	+	+	−
Propionic acid	−	−	+	+	−
Chemical sensitivity assays					
D-Serine	−	+	+	−	+
Nalidixic acid	+	−	+	−	+
Lithium chloride	+	−	+	−	+
Potassium tellurite	−	+	+	−	+

*YW, yellowish white; POY, pale orange–yellow; PY, pale yellow.
†Results in accordance with those published for *S. albida* JCM 21830^T by Ding *et al.* (2009).
‡Results in accordance with those published for *S. atrocyanea* NBRC 12956^T and *S. soli* KCTC 19389^T by Zhou *et al.* (2012).
§Results in accordance with those published for *S. flava* JCM 16034^T by Zhou *et al.* (2009).

strains are >98 % for some strains, and 99.5 % as described for *S. atrocyanea* DSM 20127^T and *S. soli* CW 59^T.

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies were obtained after growing in tryptic soy broth at 28 °C for 7 days on a rotary shaker. Genomic DNA extraction and DNA–DNA hybridization of strain MUSC 117^T and closely related type strains were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Genomic DNA extraction from strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T was performed as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). The DNA–DNA relatedness values between strain MUSC 117^T and *S. atrocyanea* NBRC 12956^T (19.8 ± 4.2 %), *S. albida* JCM 21830^T (26.8 ± 1.8 %) and *S. soli* KCTC 19389^T (20.8 ± 2.7 %) were significantly lower than 70 %, the threshold value for the delineation of genomic species (Wayne *et al.*, 1987). These results supported the notion that strain MUSC 117^T represents a novel species. Rep-PCR fingerprint analysis was used to compared strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T, *S. soli* KCTC 19389^T and *S. flava* JCM 16034^T using the primer BOX-A1R (5′-CTACGGCAAGG-CGACGCTGACG-3′) (Versalovic *et al.*, 1991; Lee *et al.*, 2014c). The PCRs were performed in a final volume of 50 µl according to protocol of SolGent 2 × *Taq* Plus PCR Smart mix using a Kyratec PCR Supercycler with cycling conditions as described by Lee *et al.* (2014d). PCR products were visualized by 2 % agarose gel electrophoresis. The rep-PCR results showed that strain MUSC 117^T exhibited a unique BOX-PCR fingerprint compared with closely related type strains (Fig. S1, available in the online Supplementary Material), therefore supporting the results of DNA–DNA hybridization that strain MUSC 117^T represents a novel species.

Analysis of the peptidoglycan amino acid composition and whole-cell sugars of strain MUSC 117^T was carried out by Identification Service of the DSMZ. The analyses were carried out according to Schumann (2011). The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan of strain MUSC 117^T contained the amino acids lysine (Lys), serine (Ser), glycine (Gly), alanine (Ala), glutamic acid (Glu) and muramic acid (Mur). The molar ratio was 1.0 Lys : 0.9 Ser : 0.7 Gly : 2.5 Ala : 1.8 Glu : 0.9 Mur. The identity of these amino acids was confirmed by agreement of the GC retention time with those of authentic standards and by characteristic mass spectrometric fragment ions of the derivatives. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C,) of the peptidoglycan contained the peptides L-Ala-D-Glu, D-Glu-Gly, L-Ala-D-Ala, L-Ser-L-Ala, L-Lys-L-Ser and L-Lys-D-Ala. From these analytical data, it was concluded that strain MUSC 117^T contained the peptidoglycan type

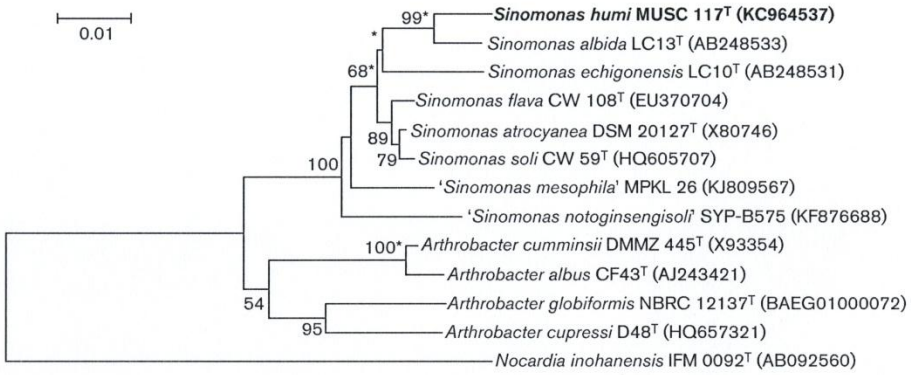

Fig. 1. Phylogenetic tree calculated using the neighbour-joining algorithm indicating the phylogenetic relationship of strain MUSC 117^T to members of species of the genus *Sinomonas*. There were a total of 1334 positions in the final dataset. Bootstrap values (>50 %) based on 1000 resampled datasets are shown at branch nodes. Bar, 10 substitutions per 1000 nucleotide positions. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm.

A3α (Schleifer & Kandler, 1972), which is consistent with the peptidoglycan type reported for type strains of species of the genus *Sinomonas* (Schleifer & Kandler, 1972; Koch *et al.*, 1995; Zhou *et al.*, 2009, 2012). The whole-cell sugars detected for strain MUSC 117^T were rhamnose, ribose, glucose, galactose and a smaller amount of mannose. Strain MUSC 117^T contained sugars such as ribose, galactose and rhamnose, which were detected in other species of the genus *Sinomonas* (Zhou *et al.*, 2009, 2012). The detection of rhamnose was noteworthy, as this sugar was not found in other species of the genus *Sinomonas*; this demonstrated a significant difference between strain MUSC 117^T and these type strains.

Cellular fatty acids analyses of strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T were carried out by the Identification Service of the DSMZ. The cell mass of strain MUSC 117^T and related type strains was harvested from tryptic soy broth after incubation at 28 °C for 2 days. The fatty acids were extracted and prepared according to the standard protocol of the MIDI (Microbial Identification) system (Sasser,

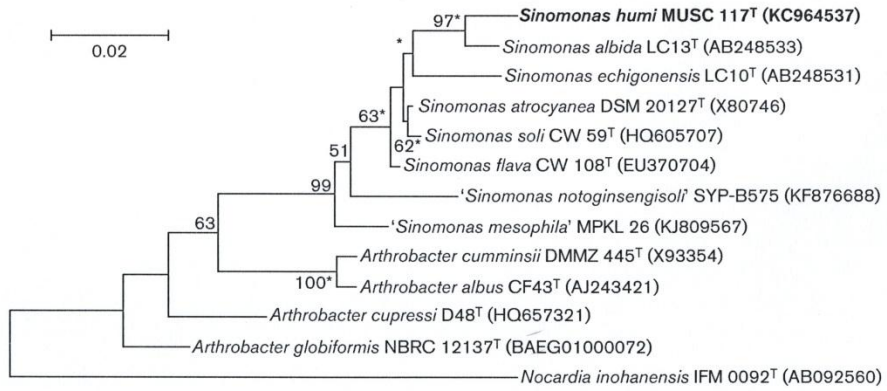

Fig. 2. Phylogenetic tree calculated using the maximum-likelihood algorithm indicating the phylogenetic relationship of strain MUSC 117^T to members of species of the genus *Sinomonas*. There were a total of 1334 positions in the final dataset. Bootstrap values (>50 %) based on 1000 resampled datasets are shown at branch nodes. Bar, 20 substitutions per 1000 nuceotide positions. Asterisks indicate that the corresponding nodes were also recovered using the neighbour-joining tree-making algorithm.

1990). The complete fatty acids profiles of strain MUSC 117^T and closely related type strains are shown in Table 2. The major cellular fatty acids were anteiso-$C_{15:0}$ (39.4 %), $C_{18:1}\omega7c$ (17.7 %), anteiso-$C_{17:0}$ (17.2 %), iso-$C_{16:0}$ (11.4 %) and iso-$C_{15:0}$ (9.8 %). Other fatty acids detected were iso-$C_{17:0}$ (1.6 %), $C_{16:0}$ (1.5 %), and trace amounts of iso-$C_{14:0}$, $C_{14:0}$ and $C_{15:0}$ (Table 2). The fatty acids profile of MUSC 117^T was consistent with those of closely related type strains such as *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T, which contained fatty acids anteiso-$C_{15:0}$ (39.4–57.4 %), anteiso-$C_{17:0}$ (8.8–22.1 %) and iso-$C_{16:0}$ (4.1–11.4 %) as their major fatty acids. However, strain MUSC 117^T contained a fatty acid, $C_{18:1}\omega7c$ (17.7 %), that was not detected in any of the related type strains (Table 2).

Analysis of respiratory quinones and polar lipids was carried out by the Identification Service of the DSMZ. The cellular polar lipids were extracted and analysed by TLC (Kates, 1986). The predominant menaquinones detected were MK-9(H_2) (86 %) and MK-9 (9 %). Minor amounts of MK-10(H_2) (1 %) and MK-8 (<1 %) were also detected. This menaquinone composition is in agreement with the report of Zhou *et al.* (2012) that the predominant menaquinone of members of the genus *Sinomonas* is MK-9(H_2). The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and five unidentified glycolipids (Fig. S2). These polar lipids are commonly found in the type strains of species of the genus *Sinomonas* (Zhou *et al.*, 2009, 2012).

Genomic DNA of strain MUSC 117^T for the determination of G+C content was extracted according to the protocol of Cashion *et al.* (1977). The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). The DNA G+C content of strain MUSC 117^T was 67.3 mol%; this is within the range of 66.9–71.8 mol% reported for species of genus *Sinomonas* (Zhou *et al.*, 2012). On the basis of phenotypic, DNA–DNA hybridization, chemotaxonomic and phylogenetic analyses, strain MUSC 117^T merits assignment to a novel species of the genus *Sinomonas*, for which the name *Sinomonas humi* sp. nov. is proposed.

Table 2. Cellular fatty acid composition of strain MUSC 117^T and closely related species of the genus *Sinomonas*

Strains: 1, *Sinomonas humi* sp. nov. MUSC 117^T; 2, *S. atrocyanea* NBRC 12956; 3, *S. albida* JCM 21830^T; 4, *S. soli* KCTC 19389^T. Data were determined in this study and represent percentages of total fatty acids. –, <0.1 % or not detected.

Fatty acid	1	2	3	4
iso-$C_{13:0}$	–	–	–	0.2
anteiso-$C_{13:0}$	–	0.1	–	0.2
iso-$C_{14:0}$	0.9	2.4	0.5	2.8
$C_{14:0}$	0.3	0.5	0.2	0.7
iso-$C_{15:0}$	9.8	11.7	17.4	19.9
anteiso-$C_{15:0}$	39.4	57.4	51.9	56.4
$C_{15:0}$	0.2	0.3	–	0.3
iso-$C_{16:0}$	11.4	10.0	4.1	7.8
$C_{16:0}$	1.5	1.9	0.7	1.8
iso-$C_{17:0}$	1.6	1.1	3.1	1.3
anteiso-$C_{17:0}$	17.2	14.7	22.1	8.8
$C_{18:1}\omega7c$	17.7	–	–	–

Description of *Sinomonas humi* sp. nov.

Sinomonas humi (hu'mi. L. gen. n. *humi* of soil, ground).

Cells stain Gram-positive and are strictly aerobic, non-motile and bent rods. Colonies are circular, convex and form yellowish-white-pigmented colonies on all different media tested. Good growth is observed on ISP2 medium, AIA, SA and nutrient agar after 7 days at 28 °C; cells grow moderately on SCA, while cells grow poorly on ISP7 medium. Using tryptic soy broth, the NaCl tolerance, temperature and pH ranges for growth are 0–4 % (w/v), 24–40 °C, pH 5.0–8.0, respectively, and optimal growth occurs at 0–2 % NaCl, 28–36 °C and pH 6–7. Cells are positive for catalase and amylase activity, but negative for cellulase, chitinase, xylanase, protease, lipase and haemolytic activities. The utilization of the following compounds as sole carbon sources can be used for differentiation from closely related type species of the genus *Sinomonas*: cellobiose, sucrose, stachyose, α-D-glucose, D-fucose, D-fructose 6-phosphate, D-aspartic acid and α-ketoglutaric acid. The following compounds are not utilized as sole carbon sources: D-salicin, D-mannitol, D-glucose 6-phosphate, gelatin, pectin, D-galacturonic acid, mucic acid, L-lactic acid, bromosuccinic acid, β-hydroxy-DL-butyric acid, acetoacetic acid and propionic acid. Sole nitrogen sources such as L-alanine are not utilized. In chemical sensitivity assays, cells are sensitive to D-serine and potassium tellurite; resistant to nalidixic acid and lithium chloride. Cells are sensitive to ampicillin, ampicillin sulbactam, cefotaxime, cefuroxime, cephalosporin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline and vancomycin. The peptidoglycan is of type A3α, containing the amino acids lysine, serine, glycine, alanine, glutamic acid and muramic acid. The whole-cell sugars are rhamnose, ribose, glucose, galactose and a smaller amount of mannose. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and five unidentified glycolipids. The major fatty acids are anteiso-$C_{15:0}$, $C_{18:1}\omega7c$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The predominant respiratory quinones are MK-9(H_2) and MK-9.

The type strain is MUSC 117^T (=DSM 29362^T=MCCC 1K00410^T=NBRC 110653^T), isolated from mangrove soil collected from the state of Pahang in Peninsular Malaysia. The genomic DNA G+C content of the type strain is 67.3 mol%.

Acknowledgements

This work was supported by a University of Malaya for High Impact Research Grant (UM-MOHE HIR Nature Microbiome Grant no. H-50001-A000027) awarded to K.-G. C., Fundamental Research Grant Scheme (FRGS/1/2013/SKK01/MUSM/03/3) and External Industry

Grant (Biotek Abadi/GBA-808813) awarded to L.-H. L. The authors are thankful to Professor Jean Euzéby for support in the Latin etymology of the novel species names.

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Chapter 5

Monashia flava gen. nov., sp. nov., a novel actinobacterium of the family *Intrasporangiaceae*

5.1 Declaration for Chapter 5

Monash University

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and conduct, data analysis, manuscript preparation	55

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Nurullhudda Zainal	Participated in experiment	NA
Nurul Syakima Ab Mutalib	Result discussion	NA
Yin Wai Fong	Result discussion	NA
Chan Kok Gan	Experimental design, result discussion, data analysis, manuscript preparation	NA
Lee Learn Han	Experimental design, result discussion, data analysis, manuscript preparation	NA

*NA – not a student registered under Monash University

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12th May 2016
Main Supervisor's Signature	[redacted]	**Date** 3rd May 2016

5.2 Summary of Chapter 5

The family *Intrasporangiaceae* was proposed and described by Stackebrandt *et al.* (1997) on the basis of the phylogenetic position of members of the genera *Intrasporangium*, *Sanguibacter*, and *Terrabacter* and a set of unique 16S rRNA gene sequence signatures. It was placed within the suborder *Micrococcineae*, order *Actinomycetales*. Later, Stackebrandt and Schumann (2000) excluded one of the genera known as *Sanguibacter* and the genera *Terracoccus* and *Janibacter* were included into the emended family *Intrasporangiaceae.* The set of signature nucleotides given originally for the family (Stackebrandt *et al.* 1997) was emended by Stackebrandt and Schumann (2000) and Zhi *et al.* (2009) also gave the signatures for the 16 genera covered. Since then, the number of newly described genera has increased and to date, the family *Intrasporangiaceae* consisted of 20 genera validly published name as *Intrasporangium* (Kalakoutskii *et al.*, 1967), *Terrabacter* (Collins *et al.*, 1989), *Terracoccus* (Prauser *et al.*, 1997), *Janibacter* (Martin *et al.*, 1997), *Ornithinicoccus* (Groth *et al.*, 1999), *Tetrasphaera* (Maszenan *et al.*, 2000), *Ornithinimicrobium* (Groth *et al.*, 2001), *Knoellia* (Groth *et al.*, 2002), *Arsenicicoccus* (Collins *et al.*, 2004), *Serinicoccus* (Yi *et al.*, 2004), *Oryzihumus* (Kageyama *et al.*, 2005), *Phycicoccus* (Lee, 2006), *Kribbia* (Jung *et al.*, 2006), *Lapillicoccus* (Lee and Lee, 2007), *Humihabitans* (Kageyama *et al.*, 2007), *Marihabitans* (Kageyama *et al.*, 2008), *Humibacillus* (Kageyama *et al.*, 2008), *Fodinibacter* (Wang *et al.*, 2009), *Ornithinibacter* (Xiao *et al.*, 2011), and *Aquipuribacter* (Tóth *et al.*, 2012). The members of *Intrasporangiaceae* have been isolated from lake and sea sediments, soil, ultrapure water, salts mines and mine waste, corals, insects, activated sludge, rotten melons, marine water, and the upper stratosphere.

In general, the members of *Intrasporangiaceae* are heterogonous with respects to their morphology (cocci, rods, short rod and mycelia), relation to oxygen (aerobic to facultative anaerobic), and the diagnostic amino acid composition of peptidoglycan where majority of the members contained either LL-diaminopimelic acid (DAP), *meso*-DAP or L-ornithine (L-Orn). The majority of the members contain menaquinones MK-8(H4) and low amount of MK-10(H4) and MK-8. They also have complex cellular fatty acids including iso-and anteiso-branched chain acids and monosaturated straight-chained acids. Majority of the family members contain similar type of polar lipids such as phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylinositol (Martin *et al.*, 1997; Maszenan *et al.*, 2000; Groth *et al.*, 2001, 2002; Hanada *et al.*, 2002; Kageyama *et al.*, 2005, 2007, 2008a, 2008b; Jung *et al.*, 2006; Lee and Lee, 2007).

The main objective in this study is to identify the taxonomic status of novel strain MUSC 78^T using a polyphasic approach and compare it with other genera of family *Intrasporangiaceae.*

The polyphasic approach results including phenotypic, genotypic, and chemotaxonomic characterization, proved that strain MUSC 78^T was different from other genera in the family *Intrasporangiaceae.* Thus, in can be concluded that strain MUSC 78^T can be classified as a novel genus in the family of *Intrasporangiaceae* and named as *Monashia* gen. nov, and its type species is named as *Monashia flava* sp. nov.

Monashia flava gen. nov., sp. nov., an actinobacterium of the family *Intrasporangiaceae*

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A novel actinobacterial strain, MUSC 78^T, was isolated from a mangrove soil collected from Peninsular Malaysia. The taxonomic status of this strain was determined using a polyphasic approach. Comparative 16S rRNA gene sequence analysis revealed that strain MUSC 78^T represented a novel lineage within the class *Actinobacteria*. Strain MUSC 78^T formed a distinct clade in the family *Intrasporangiaceae* and was related most closely to members of the genera *Terrabacter* (98.3– 96.8 % 16S rRNA gene sequence similarity), *Intrasporangium* (98.2–96.8 %), *Humibacillus* (97.2 %), *Janibacter* (97.0–95.3 %), *Terracoccus* (96.8 %), *Kribbia* (96.6 %), *Phycicoccus* (96.2–94.7 %), *Knoellia* (96.1–94.8 %), *Tetrasphaera* (96.0–94.9 %) and *Lapillicoccus* (95.9 %). Cells were irregular rod-shaped or cocci and stained Gram-positive. The cell-wall peptidoglycan type was A3γ, with LL-diaminopimelic acid as the diagnostic diamino acid. The main cell-wall sugar was mannose and lower amounts of galactose and rhamnose were present. The predominant menaquinone was MK-8(H_4). The polar lipid profile consisted of phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol and phosphoglycolipid. The predominant fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$.The DNA G+C content was 73.1 mol%. Based on this polyphasic study, MUSC 78^T exhibited phylogenetic and phenotypic differences from members of the genera of the family *Intrasporangiaceae*, and therefore a novel species of a new genus, *Monashia flava* gen. nov., sp. nov., is proposed. The type strain of *Monashia flava* is MUSC 78^T (=DSM 29621^T=MCCC 1K00454^T=NBRC 110749^T).

The family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2000) was redescribed by Zhi *et al.* (2009) and the family contains an array of actinobacteria, in addition to the type genus *Intrasporangium* (Kalakoutskii *et al.*, 1967), and has MK-8(H_4) or MK-8 as the major menaquinones and LL-diaminopimelic acid (LL-DAP), *meso*-DAP or L-ornithine (L-Orn) in the cell-wall peptidoglycan (Martin *et al.*, 1997; Maszenan *et al.*, 2000; Groth *et al.*, 2001, 2002; Hanada *et al.*, 2002; Kageyama *et al.*, 2005, 2007, 2008a, b; Jung *et al.*, 2006; Lee & Lee, 2007). At the time of writing, the family *Intrasporangiaceae* contained 19 genera, including 16 genera summarized by Zhi *et al.* (2009) and the recently described *Marihabitans* (Kageyama *et al.*, 2008b), *Fodinibacter* (Wang *et al.*, 2009) and *Ornithinibacter* (Xiao *et al.*, 2011). It is possible to distinguish these genera from each other on the basis of phenotypic and genotypic characteristics. The present investigation was designed to determine the taxonomic status of a novel actinobacterial strain, MUSC 78^T, that contained LL-DAP in the cell-wall peptidoglycan and MK-8(H_4) as the predominant menaquinone. To determine the taxonomic position of strain MUSC 78^T, a polyphasic approach was used to determine the phylogenetic, chemotaxonomic and phenotypic characteristics of the novel strain. The results indicated that strain MUSC 78^T represents a novel species of a new genus, for which the name *Monashia flavus* gen. nov., sp. nov. is proposed.

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain MUSC 78^T is KF682157.
Two supplementary figures are available with the online Supplementary Material.

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Strain MUSC 78^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located at the mangrove forests of the Tanjung Lumpur in the state of Pahang, Peninsular Malaysia, in December 2012. Samples of the upper 20 cm topsoil layer (after removing the top 2–3 cm) were collected using an aseptic metal trowel, placed in sterile plastic bags and stored in −20 °C. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C; Takahashi *et al.*, 1996). Five grams of air-dried soil was mixed with 45 ml sterilized water and ground using a mill and then the suspension was spread onto a selective isolation medium, starch casein agar (SCA; Küster & Williams, 1964) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and incubated at 28 °C for 7 days. Isolate MUSC 78^T was maintained on R2A agar medium at 28 °C and as glycerol suspensions (20 %, v/v) at −20 °C.

Cultural characteristics of strain MUSC 78^T were determined following growth on ISP 2 and ISP 7 media (Shirling & Gottlieb, 1966), SCA, *Streptomyces* agar (SA; Atlas 1993), *Actinomycetes* isolation agar (AIA; Atlas 1993) and nutrient agar (MacFaddin, 2000) for 7 days at 28 °C. The ISCC-NBS colour charts were used to determine the names and designations of colony colours (Kelly, 1964). Light microscopy (80i; Nikon) and scanning electron microscopy (JEOL-JSM 6400) were used to observe the morphologies of strains after incubation on R2A agar medium at 28 °C for 7 days. Gram staining was done following the standard Gram reaction and was confirmed by using KOH lysis (Cerny, 1978). Growth was tested at 4–44 °C at intervals of 4 °C on R2A agar medium. NaCl tolerance was tested using trypticase soy broth (TSB) and salt concentrations of 0–14 % (w/v) at intervals of 2 %. Growth was tested between pH 4.0 and 10.0 at intervals of 1 pH unit. The production of melanoid pigments and catalase activity were assessed following the protocols described by Lee *et al.* (2014a). Haemolytic activity was assessed on blood agar medium containing 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) horse blood (Carrillo *et al.*, 1996). Plates were examined for haemolysis after incubation at 32 °C for 7 days. Lipase, amylase, cellulase, chitinase, protease and xylanase activities were determined by growing cells on R2A agar medium and following the protocols described by Meena *et al.* (2013). The presence of clear zones around colonies signifies the potential of isolates for surfactant production. Antibiotic susceptibility tests were performed by the disc diffusion method as described by Shieh *et al.* (2003). Antimicrobials (Oxoid) used were as follows: ampicillin, ampicillin sulbactam, cefotaxime, cefuroxime, cephalosporin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, penicillin G, streptomycin, tetracycline and vancomycin. Cells were resistant to nalidixic acid but sensitive to the other antimicrobials used. Carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions (Biolog).

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies was obtained after growing in TSB at 28 °C for 5 days on a rotary shaker. Analysis of peptidoglycan amino-acid composition and sugars was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The analyses were performed according to published protocols (Schumann, 2011). Major diagnostic sugars of strain MUSC 78^T were obtained following the procedure described by Whiton *et al.* (1985) and analysed by TLC on cellulose plates according to Staneck & Roberts (1974). Analysis of respiratory menaquinones and polar lipids was carried out by the Identification Service of the DSMZ. The cellular polar lipids were extracted and analysed by TLC (Kates, 1986). Cellular fatty acid analyses of strain MUSC 78^T were carried out by the Identification Service of the DSMZ. Cell mass of strain MUSC 78^T was harvested from TSB after incubation at 28 °C for 5 days. The fatty acids were extracted and prepared according to the standard protocol of the MIDI Microbial Identification system (Sasser, 1990).

Genomic DNA extractions were carried out as described by Hong *et al.* (2009). PCR amplification of the 16S rRNA gene and sequencing of the purified products were done as described by Lee *et al.* (2014b). The 16S rRNA gene sequence of strain MUSC 78^T was aligned with sequences of closely related type genera classified in the family *Intrasporangiaceae* that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL X software (Thompson *et al.*, 1997). The alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic tree. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms with MEGA version 6.0 (Tamura *et al.*, 2013). Calculations of levels of sequence similarity were carried out using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980). The genomic DNA of strain MUSC 78^T for the determination of G+C content was extracted according to Cashion *et al.* (1977). The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989).

Strain MUSC 78^T formed Gram-stain-positive, non-motile, aerobic, non-spore-forming cocci or irregular rod-shaped cells (Fig. 1). Cells formed yellowish-white-pigmented colonies on ISP 2 medium and AIA. Good growth was observed on R2A agar medium, ISP 2 medium, ISP 7 medium, Luria–Bertani agar, nutrient agar and SA after 7 days at 28 °C, while cells grew moderately on AIA and SCA. Cells were positive for catalase and haemolytic activities. Strain MUSC 78^T was positive for hydrolysis of chitin, soluble starch, casein and CM-cellulose, but negative for hydrolysis of tributyrin (lipase) and xylan. Using TSB (without NaCl), the NaCl tolerance, temperature and pH ranges for growth were 0–4 %, 24–36 °C and pH 6.0–8.0

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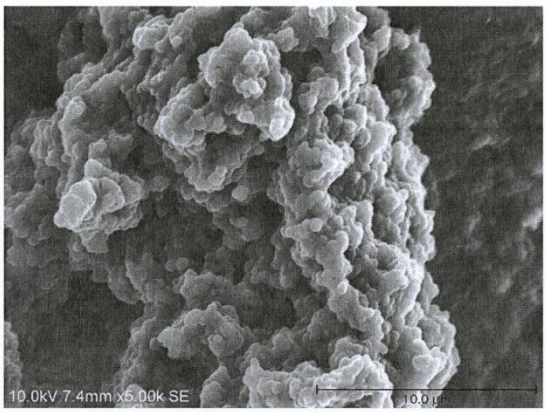

Fig. 1. Scanning electron micrograph of cells from a 5 day old culture of strain MUSC 78^T grown at 28 °C on R2A agar medium. Bar, 10 µm.

and optimal growth occurred at 0–2 % NaCl, 28–32 °C and pH 7.0. The morphological, cultural and physiological properties of strain MUSC 78^T are given in the genus and species descriptions. The organism could be distinguished from members of the family *Intrasporangiaceae* based on several chemotaxonomic characteristics (Table 1).

The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan of strain MUSC 78^T contained LL-DAP, glycine (Gly), glutamic acid (Glu), alanine (Ala) and muramic acid (Mur). The molar ratio was 1.2 LL-DAP/3.0 Gly/1.0 Glu/1.5 Ala/0.9 Mur. The identity of these amino acids was confirmed by agreement in the GC retention time with those of authentic standards and by characteristic MS fragment ions of the derivatives. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C) of the peptidoglycan contained the peptides L-Ala–D-Glu, Gly–D-Glu, Gly–D-Ala, Gly3, LL-DAP–D-Ala and LL-DAP–Gly. These data indicated that strain MUSC 78^T contains the peptidoglycan type A3γ (Schleifer & Kandler, 1972). The cell-wall sugars of strain MUSC 78^T were mannose and (at lower amounts) galactose and rhamnose. The menaquinones consisted of MK-8(H_4) (80 %), MK-8 (1 %) and MK-8(H_2) (1 %). The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphoglycolipid (Fig. S1, available in the online Supplementary Material). The major fatty acids of strain MUSC 78^T were iso-$C_{15:0}$ (34.8 %), anteiso-$C_{15:0}$ (16.7 %), iso-$C_{16:0}$ (14.5 %), iso-$C_{14:0}$ (8.6 %), anteiso-$C_{17:0}$ (4.6 %), $C_{16:0}$ (2.7 %) and $C_{18:0}$ (2.6 %) (Table 1).

The DNA G+C content of strain MUSC 78^T was 73. 1 mol%, a result that is within the range (68–74.1 %) described for members within the family *Intrasporangiaceae* (Table 1).

The nearly complete 16S rRNA gene sequence was determined for strain MUSC 78^T (1488 bp). Phylogenetic trees were reconstructed based on the 16S rRNA gene sequences (Figs 2 and S2). Comparative 16S rRNA gene sequence analysis showed that strain MUSC 78^T falls within the evolutionary radiation occupied by the family *Intrasporangiaceae* (Figs 2 and S2). The closest phylogenetic neighbours were members of the genera in the family *Intrasporangiaceae* (suborder *Micrococcineae*). Strain MUSC 78^T showed highest 16S rRNA gene sequence similarity to *Terrabacter lapilli* LR-26^T (98.3 %), *Intrasporangium oryzae* NRRL B-24470^T (98.2 %), *Humibacillus xanthopallidus* KV-663^T (97.2 %), *Janibacter anophelis* CCUG 49715^T (97.0 %), *Terracoccus luteus* DSM 44267^T (96.8 %), *Kribbia dieselivorans* N113^T (96.6 %), *Phycicoccus cremeus* V2M29^T (96.2 %), *Knoellia sinensis* DSM 12331^T (96.1 %), *Tetrasphaera japonica* ACM 5116^T (96.0 %) and *Lapillicoccus jejuensis* R-Ac013^T (95.9 %). Strain MUSC 78^T showed the closest evolutionary distance to the type strains of members of the genus *Terrabacter* at a low nucleotide sequence similarity (98.3–96.8 %). However, strain MUSC 78^T could be differentiated from the genus *Terrabacter* by the presence of anteiso-$C_{15:0}$ as a major fatty acid and the presence of phosphatidylglycerol. In addition, the genera *Intrasporangium*, *Humibacillus* and *Terracoccus*, which contain LL-DAP as the major diagnostic diamino acid in the peptidoglycan, do not contain phosphoglycolipid as a diagnostic phospholipid (Table 1). Of the other phylogenetically closely related neighbours, members of the genera *Janibacter*, *Kribbia*, *Phycicoccus*, *Knoellia*, *Tetrasphaera*, *Fodinibacter* and *Oryzihumus* are clearly different from strain MUSC 78^T as these genera contain *meso*-DAP as the diagnostic diamino acid (Table 1). Other genera such as *Ornithinibacter*, *Ornithinicoccus*, *Ornithinimicrobium* and *Serinicoccus* are different from strain MUSC 78^T as they contain L-Orn as the diagnostic diamino acid (Table 1). Differential chemotaxonomic characteristics between strain MUSC 78^T and other genera belonging to the family *Intrasporangiaceae* are summarized in Table 1. Therefore, on the basis of phylogenetic data and differential chemotaxonomic characteristics, strain MUSC 78^T does not belong to any existing genera in the family *Intrasporangiaceae*.

Based on its distinct phylogenetic position within the family *Intrasporangiaceae*, together with characteristic cell morphology, and chemotaxonomic and physiological properties, strain MUSC 78^T should be classified as representing a novel species in a new genus of the family *Intrasporangiaceae*, for which the name *Monashia flavus* gen. nov., sp. nov. is proposed.

Description of *Monashia* gen. nov.

Monashia gen. nov. (Mo.na'shi.a. N.L. fem. n. *Monashia* from Monash University).

Aerobic, non-motile, non-spore-forming, Gram-stain-positive actinobacterium of irregular coccoid to short rod-shaped cells. The predominant menaquinone is MK-8(H_4). The polar lipids are phosphatidylglycerol,

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Table 1. Differential characteristics between strain MUSC 78^T and genera of the family *Intrasporangiaceae*

Taxa: 1, strain MUSC 78^T; 2, *Terrabacter* (data from Collins *et al.*, 1989, unless otherwise indicated); 3, *Intrasporangium* (Schumann *et al.*, 1997); 4, *Humibacillus* (Kageyama *et al.*, 2008a); 5, *Janibacter* (Martin *et al.*, 1997, unless otherwise indicated); 6, *Terracoccus* (Prauser *et al.*, 1997); 7, *Kribbia* (Jung *et al.*, 2006); 8, *Phycicoccus* (Lee, 2006); 9, *Knoellia* (Groth *et al.*, 2002); 10, *Tetrasphaera* (Maszenan *et al.*, 2000); 11, *Lapillicoccus* (Lee & Lee, 2007); 12, *Arsenicicoccus* (Collins *et al.*, 2004); 13, *Ornithinibacter* (Xiao *et al.*, 2011), 14, *Ornithinicoccus* (Groth *et al.*, 1999); 15, *Ornithinimicrobium* (Groth *et al.*, 2001); 16; *Serinicoccus* (Yi *et al.*, 2004); 17, *Fodinibacter* (Wang *et al.*, 2009); 18, *Marihabitans* (Kageyama *et al.*, 2008b); 19, *Oryzihumus* (Kageyama *et al.*, 2005). ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Cell morphology	Irregular rods to cocci	Irregular rods	Hyphae	Irregular rods	Coccoid to rod-shaped	Cocci	Irregular short rods or cocci	Cocci	Coccoid to rod-shaped	Cocci	Cocci	Cocci	Branching hyphal forms	Cocci	Irregular and Cocci cocci		Irregular rods	Irregular short rods and cocci	Irregular rods
Wall diamino acid	LL-DAP	LL-DAP	LL-DAP	LL-DAP	*meso*-DAP	LL-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	LL-DAP	LL-DAP	L-Orn	L-Orn	L-Orn	L-Orn	*meso*-DAP	*meso*-DAP	*meso*-DAP
DNA G+C content (mol%)	73.1	71–73	68	69–70	70	73	69–70	74	68–69	68–71	74.1	72	69.6	72	70–71	72	72	70	72–73
Major menaquinones(s)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)	MK-8(H$_4$)
Major fatty acids*	i-C$_{15:0}$ ai-C$_{15:0}$ i-C$_{16:0}$	i-C$_{15:0}$ i-C$_{14:0}$ i-C$_{16:1}$ω7†	i-C$_{15:0}$ ai-C$_{15:0}$ i-C$_{16:0}$	i-C$_{15:0}$ iso-C$_{16:0}$ C$_{16:0}$ i-C$_{14:0}$ C$_{17:1}$ω8c	i-C$_{16:0}$ C$_{17:1}$ω8c C$_{18:1}$ω9c C$_{17:0}$‡	i-C$_{15:0}$ ai-C$_{15:0}$ C$_{16:0}$	10-Me C$_{18:0}$ i-C$_{16:0}$ C$_{18:1}$ω9c C$_{16:0}$ C$_{18:0}$	C$_{17:1}$ω8c C$_{16:0}$ i-C$_{15:0}$	i-C$_{15:0}$ i-C$_{17:0}$ i-C$_{16:0}$ ai-C$_{17:0}$	i-C$_{16:0}$ ai-C$_{17:0}$ i-C$_{16:1}$ω7 i-C$_{15:0}$ C$_{16:1}$ω5 i-C$_{14:0}$ i-C$_{16:0}$	i-C$_{16:0}$ C$_{17:1}$ω8c i-C$_{15:0}$	C$_{16:1}$ω7 C$_{18:1}$ω7 i-C$_{15:0}$ ai-C$_{15:0}$	i-C$_{18:1}$ω9c i-C$_{16:0}$ i-C$_{15:0}$ ai-C$_{15:0}$ C$_{17:0}$	i-C$_{15:0}$ ai-C$_{15:0}$ i-C$_{17:1}$ $_{11}$C$_{15:0}$	i-C$_{16:0}$ i-C$_{15:0}$ ai-C$_{15:0}$	i-C$_{15:0}$ i-C$_{16:0}$ i-C$_{17:1}$ω5 ai-C$_{15:0}$ ai-C$_{17:0}$	i-C$_{18:1}$ω9c C$_{16:0}$	i-C$_{16:0}$ C$_{17:1}$ω8c C$_{18:1}$ω9c	i-C$_{16:0}$ i-C$_{15:0}$ i-C$_{14:0}$
Polar lipid(s)§	DPG, PE, PG, PGL, PI	DPG, PE, PL, GL	ND	PE	DPG, PG, PI	DPG, PG, PL, PL	ND	DPG, PE, PI	DPG, PG, PE	DPG, PG, PL, APL, PL	ND	ND	DPG, PG, PE, PL	DPG, PG, PL, PS, PL	DPG, PG, PL, PL, GL	DPG, PG, PL, GL	DPG, PG, PE, PG, PI, 2PL	DPG, PG, PL, PIMs	DPG, PG, PGL, PL

*Major fatty acids are defined as constituting >10 % of the total fatty acid content: i, iso ; ai, anteiso; Me, methyl.
†Data from Montero-Barrientos *et al.* (2005).
‡Data from Kämpfer *et al.* (2006).
§APL, unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; PL, unknown phospholipid; PS, phosphatidylserine; PGL, unknown phosphoglycolipid; GL, unknown glycolipid.

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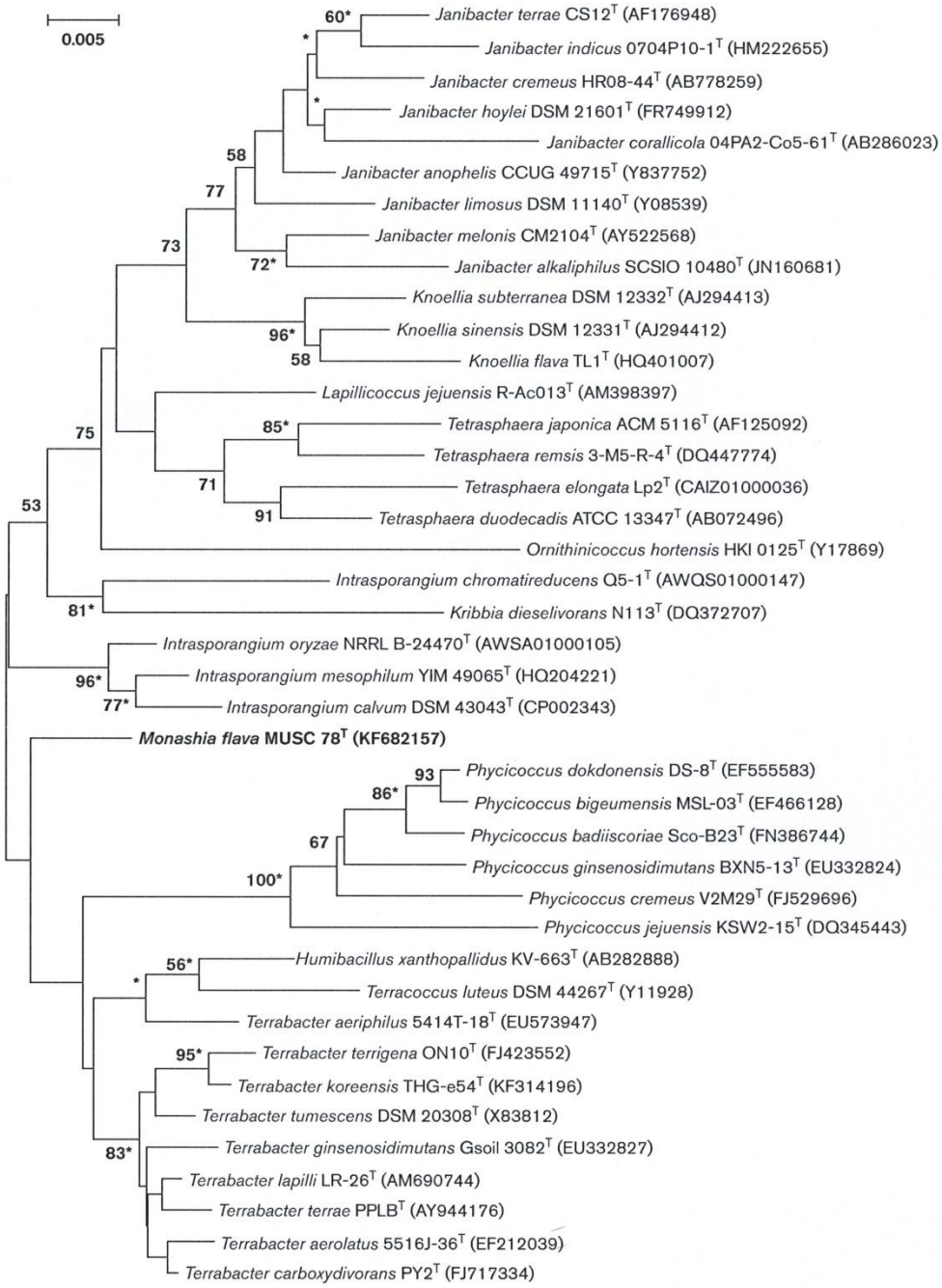

Fig. 2. Phylogenetic neighbour-joining tree (Saitou & Nei, 1987) based on 16S rRNA gene sequences of strain MUSC 78T and representatives of the family *Intrasporangiaceae*. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm. Bootstrap values (based on 1000 replicates) are shown as percentages at each node for values above 50 %. Bar, 0.005 substitutions per nucleotide position.

phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol and phosphoglycolipid. The major cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The peptidoglycan contains LL-DAP as the diagnostic diamino acid and the peptidoglycan type is A3γ. The cell-wall sugars are mannose and (at lower amounts) galactose and rhamnose.

Description of *Monashia flava* sp. nov.

Monashia flava (fla′va. l. fem. adj. *flava* yellow, referring to the colour of the colonies).

Has the following properties in addition to those given for the genus. Cells form yellowish-white-pigmented colonies on ISP 2 medium and AIA. Good growth is observed on R2A agar medium, ISP 2 medium, ISP 7 medium, Luria-Bertani agar, nutrient agar and SA after 7 days at 28 °C, while cells grow moderately well on AIA and SCA. Using TSB (without NaCl), the NaCl tolerance, temperature and pH ranges for growth are 0–4 %, 24–36 °C and pH 6.0–8.0 and optimal growth occurs at 0–2 % NaCl, 28–32 °C and pH 7.0. Cells are positive for catalase and haemolytic activities. Positive for hydrolysis of chitin, soluble starch, casein and CM-cellulose, but negative for hydrolysis of tributyrin (lipase) and xylan. The following compounds are utilized as sole carbon sources: dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, α-lactose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-arabitol, D-glucose 6-phosphate, D-fructose 6-phosphate, gelatin, glycyl L-proline, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxyphenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, citric acid, D-malic acid, L-malic acid, bromosuccinic acid, Tween 40, γ-aminobutyric acid, α-hydroxybutyric acid, α-ketobutyric acid, acetoacetic acid, acetic acid and formic acid. The following compounds are not utilized as sole carbon sources: melibiose, 3-methyl glucose, D-fucose, D-mannitol, *myo*-inositol, glycerol, D-aspartic acid, D-serine, pectin, L-galactonic acid lactone, L-lactic acid, α-ketoglutaric acid, β-hydroxy-DL-butyric acid and propionic acid. Sole nitrogen sources such as L-alanine, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid and L-serine are utilized. L-Glutamic acid is not utilized as sole nitrogen source. In chemical sensitivity assays, cells are resistant towards chemicals including fusidic acid, D-serine, troleandomycin, minocycline, lincomycin, guanine HCl, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue and sodium bromate, 1 % sodium lactate, rifamycin RV, nalidixic acid, lithium chloride, potassium tellurite, aztreonam and sodium butyrate.

The type strain is MUSC 78^T (=DSM 29621^T=MCCC 1K00454^T=NBRC 110749^T), which was isolated from mangrove soil collected from the Kuantan, the city of Pahang State in Peninsular Malaysia. The G+C content of the genomic DNA of the type strain is 73.1 mol%.

Acknowledgements

This work was supported by the High Impact Research Grant from the University of Malaya (UM-MOHE HIR Nature Microbiome Grant No. H-50001-A000027) to C. K.-G. and External Industry Grant (Biotek Abadi - Vote No. GBA-808138), Fundamental Research Grant Scheme (FRGS/1/2013/SKK01/MUSM/03/3), MOSTI Science-Fund Grant (Project No. 06-02-10-SF0300) awarded to L.-H. L. We thank Drs Peter Schumann, Susanne Verbarg and Brian Tindall from DSMZ for performing the chemotaxonomic analyses. We are grateful to Professor Dr Bernhard Schink and Dr Aharon Oren for help with the Latin etymology of the new genus name.

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Chapter 6

Antibacterial, anticancer, and neuroprotective activities of rare actinobacteria from mangrove forest soils

6.1 Declaration for Chapter 6

Monash University

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and conduct, data analysis, manuscript preparation	75

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Iekhsan Othman	Result discussion	NA
Fang Chee Mun	Result discussion	NA
Chan Kok Gan	Result discussion	NA
Goh Bey Hing	Experimental design, result discussion, data analysis, manuscript preparation	NA
Lee Learn Han	Experimental design, result discussion, data analysis, manuscript preparation	NA

*NA – not a student registered under Monash University

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12th May 2016
Main Supervisor's Signature	[redacted]	**Date** 3rd May 2016

6.2 Summary of Chapter 6

The phylum *Actinobacteria* have been studied and screened since the 1950s as they produce a wide range of biological activities such as antimicrobial, anticancer, antifungal, antivirus, cytotoxic and immunosuppressive agents (Manivasagan *et al*., 2014). Actinobacteria such as *Streptomyces* is a prolific producer of bioactive compound especially secondary metabolites since this genus have been produced about 7600 compounds (Berdy, 2005). However, frequent rediscovery of *Streptomyces* has reduced the chance of finding new bioactive compounds from this species (Hong *et al*. 2009; Hamedi *et al*. 2015). According to Hwang *et al*. (2014), the isolation of new antibiotic from *Streptomyces* has declined to about 20% to 30%. At the same time, disease including infectious disease (Talbot *et al*., 2006) and life-threatening diseases such as cancer (Olano *et al*., 2009) are continuously developing resistance towards existing drugs causing the urgent need for discovering of new compounds for drug development (Neha and Sandeep, 2014).

Recently, there is evidence that rare actinobacteria could represent a unique source of novel bioactive compounds. The rare actinobacteria strain from different genera namely *Amycolatopsis, Saccharopolyspora, Actinoplanes* and *Micromonospora* have been proven as excellent sources of novel bioactive compounds (Subramani and Aalbersberg 2013; Azman *et al*. 2015a). Moreover, rare actinobacteria from unexplored ecosystem such as mangrove has gained attention from the researcher for novel strains and novel bioactive compound discovery due to its unique condition (Xu *et al*. 2014) where it situated at the interphase between the terrestrial and marine environment (Ghosh *et al*., 2010).

The isolation and identification of novel rare actinobacteria by polyphasic approach from mangrove soils in Tanjung Lumpur, Peninsular Malaysia namely *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T} (Lee *et al*. 2014; Lee *et al*. 2015; Azman *et al*. 2015b) has led to the investigation of the bioactive compounds on different bioactivities. Therefore, the main objective of this study is to screen the antibacterial, neuroprotective and cytotoxic activities of the *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T} extracts.

Overall, results from this study provided evidences that the *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T} extracts have good antibacterial properties but selective neuroprotective and cytotoxic activities. Therefore, these three species clearly merit for further investigation in identifying the bioactive principles and the mechanism of the bioactivity.

Antibacterial, anticancer and neuroprotective activities of rare actinobacteria from mangrove forest soils

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Mangrove is a complex ecosystem that contains diverse microbial communities, including rare actinobacteria with great potential to produce bioactive compounds. To date, bioactive compounds extracted from mangrove rare actinobacteria have demonstrated diverse biological activities. The discovery of three novel rare actinobacteria by polyphasic approach, namely *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T} from mangrove soils at Tanjung Lumpur, Peninsular Malaysia have led to the screening on antibacterial, anticancer and neuroprotective activties. A total of ten different panels of bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, ATCC 70069, *Pseudomonas aeruginosa* NRBC 112582 and others were selected for antibacterial screening. Three different neuroprotective models (hypoxia, oxidative stress, dementia) were done using SHSY5Y neuronal cells while two human cancer cells lines, namely human colon cancer cell lines (HT-29) and human cervical carcinoma cell lines (Ca Ski) were utilized for anticancer activity. The result revealed that all extracts exhibited bacteriostatic effects on the bacteria tested. On the other hand, the neuroprotective studies demonstrated *Microbacterium mangrovi* MUSC 115^{T} extract exhibited significant neuroprotection properties in oxidative stress and dementia model while the extract of strain *Monashia flava* MUSC 78^{T} was able to protect the SHSY5Y neuronal cells in hypoxia model. Furthermore, the extracts of *Microbacterium mangrovi* MUSC 115^{T} and *Monashia flava* MUSC 78^{T} exhibited anticancer effect against Ca Ski cell line. The chemical analysis of the extracts through GC-MS revealed that the majority of the compounds present in all extracts are heterocyclic organic compound that could explain for the observed bioactivities. Therefore, the results obtained in this study suggested that rare actinobacteria discovered from mangrove environment could be potential sources of antibacterial, anticancer and neuroprotective agents.

Keywords: *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T}, *Monashia flava* MUSC 78^{T}, antibacterial, anticancer, neuroprotective

Actinobacteria is common soil inhabitant and have a high proportion of total microbial biomass in soil (1). They are considered as the most economically significant as well as biotechnologically valuable microbe, producing bioactive compounds including antibiotics, antimicrobial, anticancer, antitumor, enzyme, enzyme inhibitors and immunosuppressive agents

(2). Actinobacteria such as *Streptomyces* are excellent producer of bioactive compounds especially secondary metabolites (3-8). Over 10, 000 bioactive compounds were derived from actinobacteria species, 7600 (76%) compounds were derived from *Streptomyces* while 2400 (24%) compounds isolated from rare actinobacteria (9). Unfortunately, repeated isolation of known compounds and a reduced hit-rate of novel compounds have limited the development of new and effective drugs to treat ever increasing human diseases. (10-12). At the same time, the arising multi drug resistance (MDR) pathogen and other deadly diseases caused the dramatic increase in demand to look for new compounds (13) from other sources such as rare actinobacteria.

Previously, the numbers of rare actinobacteria being discovered were low, as compared to *Streptomyces*. This is due to the facts that they are difficult to isolate, cultivate and maintain under conventional conditions (14). However, the number of novel rare actinobacteria is increasing, from only 11 genera in 1970 to 220 genera by 2010 (15). At the time of writing (March 2016), there are approximately 340 genera of rare actinobacteria (www.bacterio.net) discovered from various environment thus demonstrating that rare actinobacteria are widely distributed in the biosphere. According to Goodfellow (2010) (16), there are a low number of rare actinobacteria isolated from marine environments such as the mangrove. Thus, mangrove environment has gained attention from the researcher due to its location where it situated at the inter-phase between the terrestrial and marine environment, and have a special condition such as high moisture, high salinity and hypoxia tolerant (17). This condition breeds many novel microorganism including rare actinobacteria that contained special and unique metabolic pathways to adapt with those conditions and lead to the production of valuable metabolites (17).

The rare actinobacteria strains discovered in previous work were *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T}. These strains were isolated from mangrove soils located at Tanjung Lumpur, Peninsular Malaysia. The taxonomic status of these strains was described in previous publications using polyphasic approach (18-20). Currently, the study of bioactive compounds from mangrove rare actinobacteria become popular as they possess great potential to pharmaceutical industry (3,10,14). An example, Mangamuri et al. (2015) (21) reported that the bioactive metabolites from

Pseudonocardia endophytica VUK-10 was able to inhibit the growth of Gram-positive and Gram-negative bacteria, yeast, fungi and also exhibited potent cytotoxic activity against human breast adenocarcinoma cell line (MDA-MB-231, MCF-7), human cervical cell line (HeLa), human ovarian cyst adenocarcinoma cell line (OAW-42). Janardhan et al. (2014) (22) showed the extracts of strain *Nocardiopsis alba* isolated from mangrove soil of Nellore regions, Andhra Pradesh, India, exhibited potent total antioxidant property. Novel anticancer and anti-infection compounds are being isolated from mangrove rare actinobacteria, as represented by the discovery of the salinosporomide A, an anti-cancer compound produced by *Salinispora tropica* (23).

In light of this, the present study was initiated to investigate the extracts of rare actinobacteria collected from Tanjung Lumpur, Peninsular Malaysia for its biological activity such as antibacterial, anticancer or neuroprotective activity.

2.0 MATERIALS AND METHODS

2.1 Preparation of Microbacterium mangrovi MUSC 115^T, Sinomonas humi MUSC 117^T and Monashia flava MUSC 78^T extracts

All novel strains were grown on ISP2 medium for 5 days prior to fermentation process. The fermentation medium used was FM3 (10, 24) with slight modification and autoclaved at 121°C for 15 minutes. The strains were cultured at 200 rpm, for 7-10 days at 28^{o}C. The resulting fermentation media were separated from the mycelium by centrifugation at 4500 rpm at 4^{o}C for 30 minutes. The supernatant was collected and subjected to freeze dry process. Upon freeze-drying, the sample was extracted with methanol for 72 hours (ratio 3:1; methanol:sample) and the residue was re-extracted under the same condition twice at 24 h interval with ratio of 2:1 and 1:1, respectively. All the methanol-containing extract was filtered and evaporated using a rotary vacuum evaporator and the extract were kept in -20^{o}C until further analysis (25).

2.2 Bacterial strains

Ten different pathogens were used for the antibacterial screening; namely *Acinetobacter calcoaceticus* NBRC 13006, *Salmonella typhi* ATCC 19430, *Escherichia coli* ATCC 25922, *Vibrio parahaemolyticus* VP103 (Jeffrey Cheah School of Medicine and Health Science laboratory), *Pseudomonas aeruginosa* NRBC 11258, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, ATCC 70069, ATCC 33591, ATCC BAA-44, *Bacillus subtilis* ATCC 31098. The test organisms were maintained on Mueller-Hinton agar (MHA).

2.3 Minimal inhibitory concentration (MIC) determination

Minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that able to inhibit the growth of particular bacterium after overnight incubation. The work was performed by the broth microdilution method in 96 well plate as described by Wiegand, Hilpert & Hancock (2008) (26), with slight modification. Chloramphenicol (0.1mg/mL) was used as positive control and untreated bacterial culture was used as negative control. One hundred microliters aliquot of the bacteria was added into the wells with an approximate inoculum of 1 x 10^6 CFU/mL, previously prepared as a 0.5 McFarland's standard. Serial dilutions of the extracts were done to achieve the final concentration of 5, 2.5, 1.25, 0.625 and 0.313 mg/mL. Aliquot (100 uL) of the extract with different concentration was added into each of the wells and incubated at 37^{o}C for 24 hours. The MIC was determined by assessment of turbidity by optical density readings at 600nm.

2.4 Minimal bactericidal concentration (MBC) determination

Minimal bactericidal concentration (MBC) is the lowest concentration of an antimicrobial that prevent the growth of particular microorganism. The MBC was determined by sub-culturing 100 µL from well that exhibited no growth onto MHA and incubated at 37^{o}C for 24 hours.

2.5 Cell lines maintenance and growth condition

The human cancer cell lines (HT-29 and Ca Ski) and the neuronal cell lines (SH-SY5Y) involved in this study was maintained in Roswell Park Memorial Institute (RPMI) and

Dulbecco's Modified Eangle Medium (DMEM), respectively, supplemented with 10% fetal bovine serum and 1X antibiotic-antimycotic at 37°C humidified incubator containing 5% CO_2 (6).

2.6 Neuroprotective assay

Cell viability of neuronal cells were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, SHSY-5Y cells were seeded into a microtitre plate at a density of $3x10^4$ cells/well and allowed to adhere overnight. 20 µL of each extracts were added into the cells with the final concentration ranging from 6.25 to 200 µg/mL. Catechin (100 µM) (oxidative stress and hypoxia model) or gallic acid (1 µg/mL) (dementia model) were used as a positive control in the experiments of the study. The pre-treated cells were incubated for 2 hours followed by either 250 µM hydrogen peroxide (H_2O_2), 400 µM streptozotocin (STZ) or 5 mM cobalt (II) chloride ($CoCl_2$) treatment for 24 hours.

2.7 Anticancer activity of the extract on human cancerous cells

The effect of extracts on cell viability of human cancer cells lines was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into a microtitre plate at a density of $5x10^3$ cells/well and allowed to adhere overnight. 20 µL of each extracts were added into the wells with the final concentration ranging from 6.25 to 200 µg/mL and incubated at 72 hours. Curcumin (3 µg/mL) was included as positive control.

2.8 MTT assays

The MTT assay for neuroprotective and cytotoxic activities were performed by adding 20 uL of MTT (5 mg/mL) into each well and the plates were incubated at 37^oC containing 5% CO_2 for 4 hours (25). After the incubation period, the medium was then aspirated carefully and 100 uL of DMSO was added. The absorbance of the product was determined spectrophotometrically at 570 nm, with 650 nm as reference using a microplate reader. The percentage of cell viability was calculated as follows:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \quad X\ 100\%$$

2.9 Statistical analysis

All values expressed as mean ± standard deviations (SD) by Microsoft Excel. Data were analyzed for statistical significance using one-way ANOVA, followed by Dunnett's test as a post-hoc test with GraphPad Prism 6.0 software for Windows (Inc., San Diego, USA).

2.10 Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was performed in accordance with our previous developed method with slight modification (27, 28). The instrument used was Agilent Technologies 6980N (GC) equipped with 5979 Mass Selective Detector (MS), HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0m x 250µm and helium as carrier gas at 1 mL/min. The column temperature was programmed initially at 40^{o}C for 10 minutes, followed by an increase of 3^{o}C/minutes to 250^{o}C and was kept isothermally for 5 minutes. The MS was operating at 70 eV. The constituents were identified by comparison of their mass spectral data with those from NIST 05 Spectral Library.

3.0 RESULTS AND DISCUSSIONS

3.1 Antibacterial assays

Results of antibacterial activity are represented in Table 1. Showed that all extracts demonstrated good inhibitory activity against different bacteria tested, with MICs values of 2.5 mg/mL, except for *P. aeruginosa* NRBC 112582, *V. parahaemolyticus* VP103 and *E. coli* ATCC 25922, where the inhibitory concentration was 1.25 mg/mL. These finding indicates that the extracts inhibited the growth of the bacteria tested at low concentrations.

The MBC result in Table 1 presented *Microbacterium mangrovi* MUSC 115^{T} and *Sinomonas humi* MUSC 117^{T} extracts were completely inhibiting the growth of *P. aeruginosa* NRBC 112582, *S. typhi* ATCC 19430 and *E. coli* ATCC 25922 at a concentration of 5 mg/mL.

However, a higher concentration might be needed for complete inhibition of the growth of the other bacteria tested.

In addition, some of the extracts displayed bactericidal effects on few numbers of bacteria. According to Ocampo et al. (2014) (29), bacteriostatic can be defined as the agent inhibit the growth of bacteria without killing effects, while bactericidal means agents that kill bacteria. An extract is considered as bactericidal when the ratio of MBC/MIC is ≤ 4 and bacteriostatic when this ratio is > 4 (30). This effect was observed with the *Microbacterium mangrovi* MUSC 115^T extract against *P. aeruginosa* NRBC 112582, *S. typhi* ATCC 19430, and *E. coli* ATCC 25922 with the ratios of MBC/MIC equal to 4, 2, and 2 respectively. The *Sinomonas humi* MUSC 117^T extract was also bactericidal against E. *coli* ATCC 25922 with the ratio of MBC/MIC equal to 2. Overall, the extracts of each strains possessed activity to inhibit the growth of bacteria tested.

Table 1: Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MBC/MIC ratios of the extracts of strains *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T

Bacterium tested	**MUSC 115^T**			**MUSC 117^T**			**MUSC 78^T**		
	MIC	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**
**Staphylococcus aureus* ATCC 43300	2.5	>5	-	2.5	>5	-	2.5	>5	-
**Staphylococcus aureus* ATCC 70069	2.5	>5	-	2.5	>5	-	2.5	>5	-
**Staphylococcus aureus* ATCC 33591	2.5	>5	-	2.5	>5	-	2.5	>5	-
**Staphylococcus aureus* ATCC BAA-44	2.5	>5	-	2.5	>5	-	2.5	>5	-
Acinetobacter calcoaceticus NBRC 13006	2.5	>5	-	2.5	>5	-	2.5	>5	-
Bacillus subtilis ATCC 31098	2.5	>5	-	2.5	>5	-	2.5	>5	-
Pseudomonas aeruginosa NRBC 112582	1.25	5	4	1.25	>5	-	1.25	>5	-
Salmonella typhi ATCC 19430	2.5	5	2	2.5	>5	-	2.5	>5	-
Vibrio parahaemolyticus VP103	1.25	>5	-	1.25	>5	-	1.25	>5	-
Escherichia coli ATCC 25922	2.5	5	2	1.25	5	2	1.25	>5	-

*Methicillin-resistant *Staphylococcus aureus* (MRSA)
*(-): not calculated for MBC/MIC as the MBC value was >5 mg/mL

3.2 Neuroprotective assays

In this study, the neuroprotective assays were performed by using three different experimental models focusing on hypoxia, oxidative stress and dementia. Results of each experimental model were show in Figure 1, 2 and 3, respectively.

3.2.1 Neuroprotection properties of extracts on hypoxia induced cytotoxicity

Hypoxia can be defined as the reduction or lack of oxygen in organs, tissues or cells. A common experimental model of hypoxia was created using a transition metal, cobalt (II) chloride ($CoCl_2$) (31-32). $CoCl_2$ is a chemical agent that reportedly induces a biochemical and molecular response similar to that observed under low-oxygen conditions in mammalian systems (33). Beside it is widely used to establish the model of hypoxia in both *in vitro* and *in vivo* study. Theoretically, the Co^{2+} will replace the Fe^{2+} in heme on the cell surface, thus weaken the oxygen signaling and transport, leading to the generation of reactive oxygen species (ROS) and cell death (34). In fact, a study done by Lee, Lee and Rho (2001) (35) and Vengellur and LaPres (2004) (36) have shown that both hypoxia and cobalt affecting a similar group of genes on a global gene expression level. This implies that the robustness and suitability use of this model of experiment for experimental purposes.

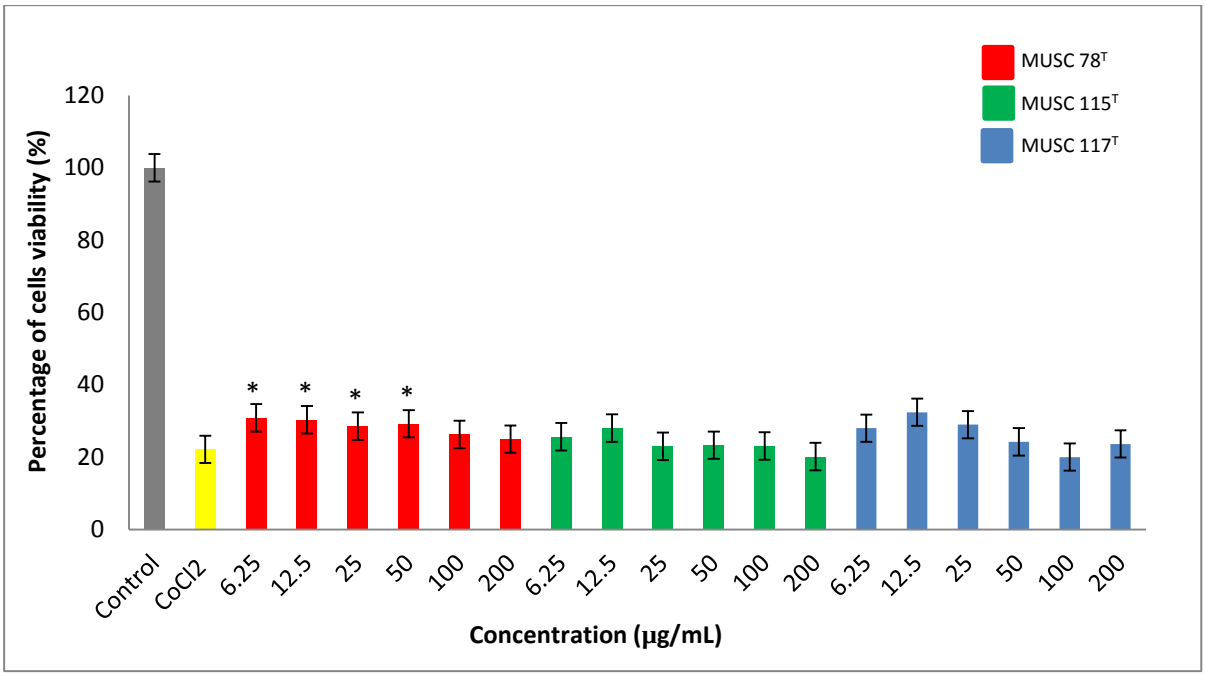

Figure 1: The neuroprotective activity of methanolic extracts on the cell viability of SH-SY5Y cells treated with $CoCl_2$. Cells viability was measured using MTT assay. *$p<0.05$ indicates statistically significant differences compared to $CoCl_2$ induced cells.

Figure 1 showed that the neuronal cells subjected to $CoCl_2$ exposure showed a significant reduction in viability of cells up to 77.8%. Based on the analysis, the extract of *Monashia flava* MUSC 78^T was able to protect the neuronal cells from the $CoCl_2$ insult at lower concentration; 6.25 to 50 µg/mL. The neuroprotective activity reduced when the concentration of the extracts reached at 50 µg/mL. The statistical analysis of *Sinomonas humi* MUSC 117^T and *Microbacterium mangrovi* MUSC 115^T extracts showed the percentages of cell viability for each concentration tested were not significant when compared to $CoCl_2$ induced cells and concluded that these two extracts were not able to protect neuronal cells from the hypoxia induced neuronal damage.

3.2.2 Neuroprotection properties of extracts on oxidative stress induced cytotoxicity

One of the most common methods applied for studying the *in vitro* neuroprotective activity of antioxidants is H_2O_2 induced cytotoxicity (37-38), hence this method was employed to study the extracts of each strain. The insults of H_2O_2 have been linked to the formation of oxidative stress which is known to cause neurodegenerative diseases such as Alzheimer's (39) and Parkinson diseases (40). H_2O_2 has a short half-life, and its dissociation into hydroxyl and superoxide ions may affect the membrane integrity and leading to cellular

damage (39, 41). In fact, H_2O_2 has been observed to exert toxic effect on different cell types while neuron was found to be most susceptible to H_2O_2-induced toxicity (42).

Based on Figure 2, it was observed that only *Microbacterium mangrovi* MUSC 115^T extract was able to protect the neuronal cells against H_2O_2 challenge at low concentration, 6.25 µg/mL. Furthermore, there is a significant decreased in cell viability in *Microbacterium mangrovi* MUSC 115^T extract treated cells from 50 µg/mL to 200 µg/mL as compared to H_2O_2 control. This indicates the effect of the treatment reached its maximum efficacy at around 12.5 µg/mL. Further increase of treatment will eventually found to be toxic towards the neuronal cells. On the other hand, the *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T extracts were found to exhibit no protective activity on SH-SY5Y neuronal cells when challenged by H_2O_2.

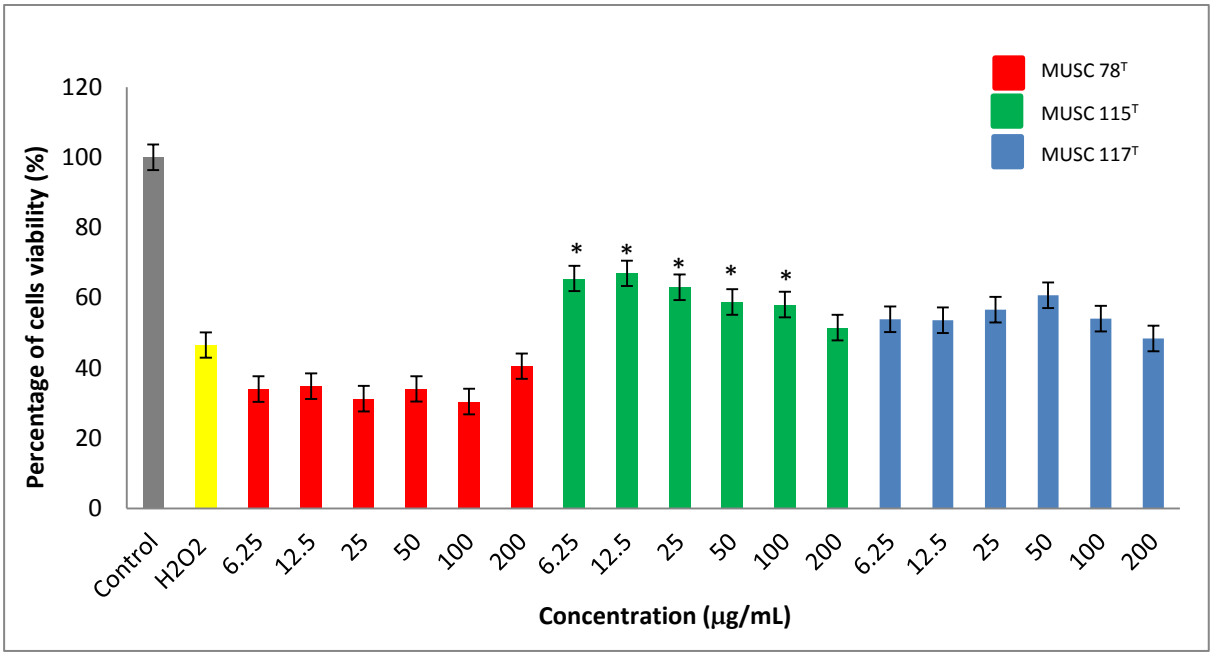

Figure 2: The neuroprotective activity of methanolic extracts on the cell viability of SH-SY5Y cells treated with H_2O_2. Cells viability was measured using MTT assay. *$p<0.05$ indicates statistically significant differences compared to H_2O_2 induced cells.

3.2.3 Neuroprotection properties of extracts on dementia induced cytotoxicity

Dementia is known as a multisystem-related neurodegenerative disorder. A set of symptoms are associated to this disease which include impairment in short- and long-term memory, impairment in thinking, judgment, other disturbance of higher cortical function, or

personality change (43). In order to understand the pathological aspect of dementia in human, researcher have made use of STZ as an inducer in rats to create the experimental model of dementia (44). It was also commonly utilized in preparing the *in vitro* dementia model of experiment particularly on SH-SY5Y neuronal cells (45). The induction of STZ was found to generate excessive free radicals which leading to formation of oxidative stress (46), inflammation (47), abnormal protein (48) and leads to mitochondrial dysfunction and apoptosis in cell (49).

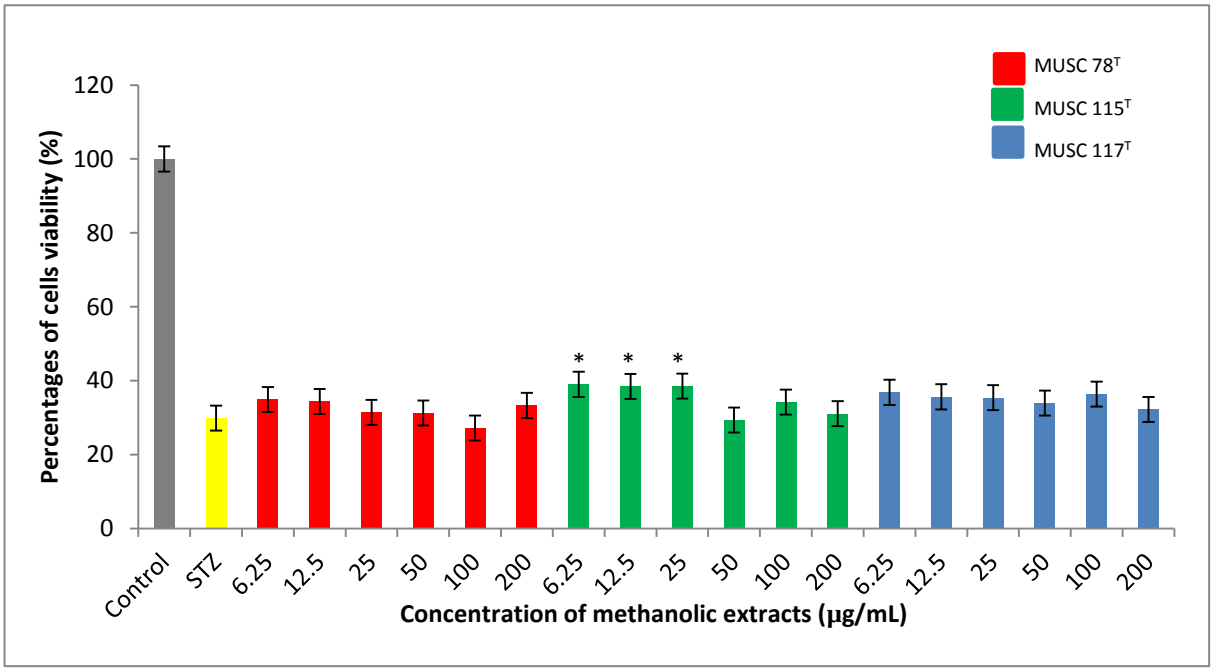

Figure 3: The neuroprotective activity of methanolic extracts on the cell viability of SH-SY5Y cells treated with STZ. Cells viability was measured using MTT assay. *$p<0.05$ indicates statistically significant differences compared to STZ induced cells.

Figure 3 demonstrated the result of neuroprotective activity of extracts on dementia model of experiment. From the data, the percentage of cell viability of SH-SY5Y neuronal cells treated with STZ only was found to be significantly ($p<0.05$) reduced up to about 70.0%. The pre-treatment of *Monashia flava* MUSC 78^T and *Sinomonas humi* MUSC 117^T extracts on the STZ treated cells showed that both of these extracts were unable to protect SH-SY5Y cells from the STZ induced neuronal damage. However, only *Microbacterium mangrovi* MUSC 115^T extracts treatment was found to demonstrating neuroprotective activity at different concentration ranging from 6.25 to 25 µg/mL.

3.3 Anticancer activity of the extracts on human cancerous cells

Rare actinobacteria, represent a promising reservoir of different kinds of therapeutics drugs. In this study, the anticancer effect of the extracts were tested on two different types of human cancer cell lines; human colon cancer cell lines (HT-29) and human cervical carcinoma cell lines (Ca Ski). The effects of the extract on the tested cancerous cells are shown Figure 4 and Figure 5.

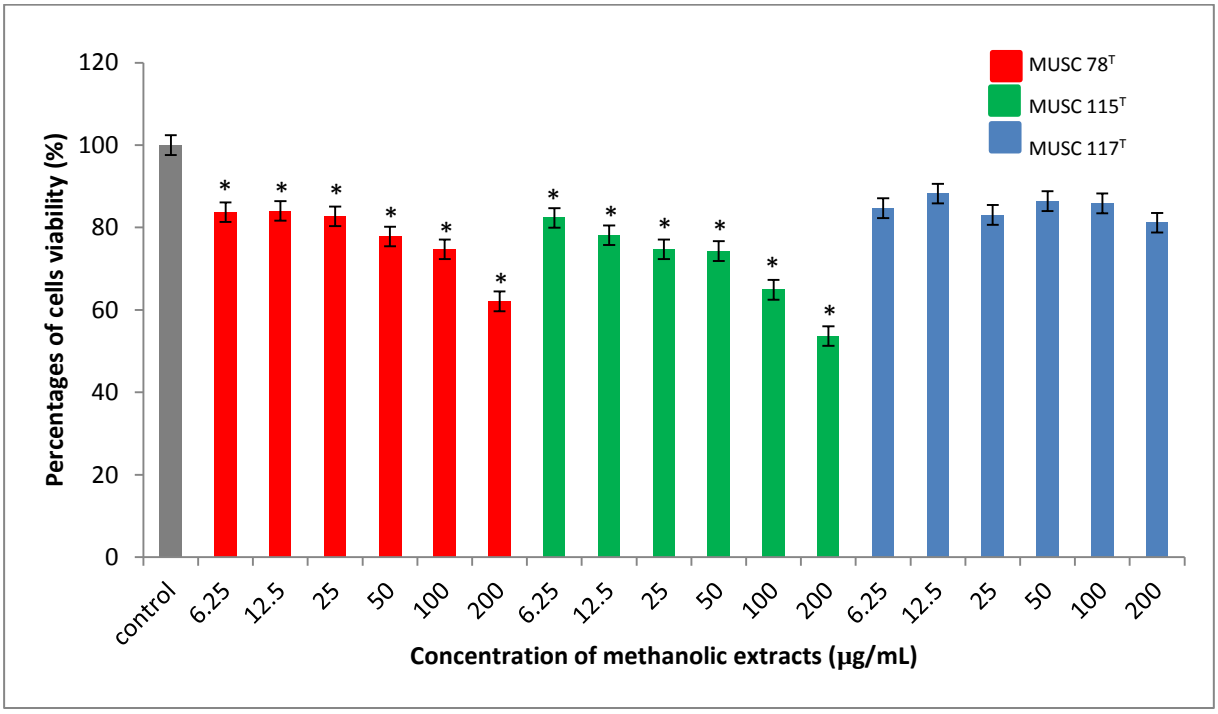

Figure 4: The anticancer activity of methanolic extracts on the cell viability of Ca Ski cells. Cells viability was measured using MTT assay. *p<0.05 indicates statistically significant differences compared to untreated cells.

All the extracts displayed varying levels of anticancer against the Ca Ski cells (Figure 4). Interestingly, a dose-dependent response was observed for the *Monashia flava* MUSC 78^T and *Microbacterium mangrovi* MUSC 115^T extracts treatment as there was a significant reduction of the viability of cells when compared to the untreated cells. The Ca Ski cells was found to be the most vulnerable to the treatment of *Microbacterium mangrovi* MUSC 115^T extracts with the strongest growth inhibition at high concentration tested (200 µg/mL), seconded by the treatment activity displayed by *Monashia flava* MUSC 78^T. In the case of *Sinomonas humi* MUSC 117^T extract, there was no significant reduction of the viability of Ca Ski cells as compared to control. Overall, *Monashia flava* MUSC 78^T and *Microbacterium mangrovi* MUSC 115^T extracts are effective in inhibiting the growth of Ca Ski cells.

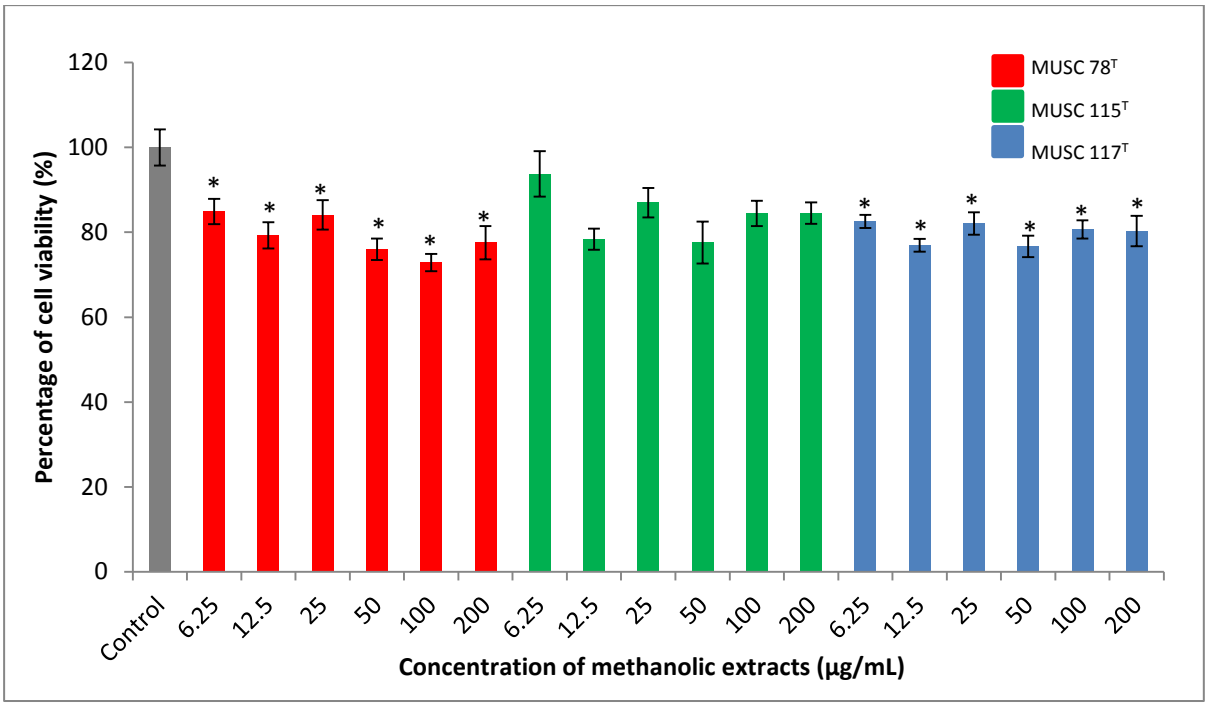

Figure 5: The anticancer activity of methanolic extracts on the cell viability of HT-29 cells. Cells viability was measured using MTT assay. *p<0.05 indicates statistically significant differences compared to untreated cells.

Similarly, the anticancer effect of *Monashia flava* MUSC 78^{T}, *Microbacterium mangrovi* MUSC 115^{T} and *Sinomonas humi* MUSC 117^{T} extracts on HT-29 cells were examined as well. Results in Figure 5 displayed that there is a mild growth inhibition activity of HT-29 cells as the viability of HT-29 cells was significantly reduced especially at the highest concentration of treatment at 200 µg/mL.

Taken altogether, the extracts of *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T} were shown to be effective in causing anticancer effect on this two different cancer cell lines namely human colon cancer cell lines (HT-29) and human cervical carcinoma cell lines (Ca Ski). The results of the studies also demonstrated that two cancer cell lines showed different reaction towards the concentration of extracts tested. *Microbacterium mangrovi* MUSC 115^{T} and *Monashia flava* MUSC 78^{T} extracts exhibiting a cytotoxic activity on Ca Ski cells except for *Sinomonas humi* MUSC 117^{T}, meanwhile all the extracts exhibiting a low anticancer activity against HT-29 cells. In general, varying strength at the effect possessed by extract are most likely affected by the differences in the chemical composition that present in.

3.4 Chemical profiling analysis

Following the assessment of bioactivities possessed by *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T extracts, GC-MS analysis were performed in order to analyze the chemical constituents that present in the extracts. GC-MS is an effective combination of technologies which meant for the analysis of chemical compounds. Basically, the compounds will be separated by GC while MS generates the characteristic mass profile for each of the compounds detected (50, 51). As shown in Table 2, a total of six chemical compounds were identified in *Microbacterium mangrovi* MUSC 115^T extract, ten compounds were detected in *Sinomonas humi* MUSC 117^T extract while *Monashia flava* MUSC 78^T extract analysis yielded a total of twenty compounds. Through GC-MS analysis, the obtained results indicating the majority of the compounds that present in extracts are consisted of organic heterocyclic compounds. These heterocyclic compounds include phenolics, pyrazines, and pyrrolopyrazine.

Phenolic are a class of organic compound consisting of a hydroxyl group bonded to an aromatic hydrocarbon group. Among other heterocyclic organic compound, phenolic compounds have attracted the attention of researchers as they are well known for their antioxidant and free radical-scavenging abilities. These potent bioactivities are associated with potential beneficial effects on human health (52). In fact, phenolic compounds have been reported to possess potent antioxidative, anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities (53-56). Through GC-MS analysis, the phenolic compound known as, 2,4-di-tert-butyl phenol (2,4 DTBP) (**2, 9, 27**) was detected in all of the extracts tested in current study. Literature has shown that 2,4 DTBP can be produced by microorganisms such as fungus (57) and bacteria (58). For example, this compound has been detected in *Pseudomonas monteilii* PsF84 and was found to be effective against *Fusarium oxysporum* (59). Besides, the existene of this compound in *Lactococcus* sp was associated to its antifungal and antioxidant properties as well as its cytotoxic activity (58). It was also reported that, the antibacterial activity of *Monochaetia kansensis* could be due to the presence of 2,4 DTBP as well (60).

Another group of compound that was found in this study was pyrazines. Pyrazines are known to exist in form of complex structure with the present of nitrogen atoms in their aromatic ring. These compounds are greatly known for their strong odor properties and have

been detected in several bacteria. The value of this group of compound reside with theirs bioactivities, as pyrazines are commonly known to exhibit antimicrobial, anticancer, antioxidant as well as neuroprotection properties (6, 61-64). In current study, the pyrazines compounds; **19, 21, 22, 23** and **24** were found in *Monashia flava* MUSC 78^T extract. Previous study has shown that compound **19** and **21** were detected in myxobacteria *Stigmatella* WXNXJ-B which known to exhibit a significant high level of antitumor activities (65). Meanwhile, compound **22**, **23** and **24** were detected previously in *Streptomyces antioxidans* and were found to have a strong antioxidant activities (64).

The complex structure form with incorporation of one or more pyrrole compound into a pyrazine is normally known as pyrrolopyrazine. The GC-MS characterization analysis have also demonstrated the existence of pyrrolopyrazine compounds in all of the extracts. For example, compounds such as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (**4**, **11**, **32**) and (3R,8aS)-3-methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione (**3**, **10**, **31**) were found as the constituents of the mixture. Literature has shown that these compounds were detected in different *Streptomyces* species (6, 64, 66) which include *Nocardia* sp. (67), and *Bacillus* sp. (68) and was associated to antioxidant activity. Another pyrrolopyrazine compound identified in MUSC 115^T & MUSC 78^T strains was 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane (BDDB) (**6**, **36**). Gohar et al. (2010) (69) have reported the present of BDDB in *Burkholderia cepacia* may responsible for the antibacterial activity against *Aeromonas hydrophila, Edwardsiella tarda* and *Vibrio ordalli*. Besides, the detection of BDDB in *Streptomyces cacaoi* GY525 (70) was believed to contribute for the mortality of second-stage juvenile and hatch inhibition of *Meloidogyne incognita*. Therefore, the detection of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (**16)** in *Sinomonas humi* MUSC 117^T and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (**35**) in *Monashia flava* MUSC 78^T might contribute for the observed bioactivities. In fact, these compounds were seen to occur in quite a number of different *Streptomcyes* species which have demonstrated to exhibit a wide range of bioactivities (6, 64, 66, 71, 72). For example, the present of these compounds in microorganism has been associated to the strong antibacterial activity against *E. coli, P. aeruginosa* and *E. faecalis* (73). Besides, Hong et al. (2008) (74) have also showed **16** was able to inhibit expression of serine/threonine kinase Akt which may be useful for inhibition of cell proliferation and activation of apoptosis activity in cancer cells. Perhaps, the detection of 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (**6**, **17**) in *Microbacterium mangrovi* MUSC 115^T and *Sinomonas humi* MUSC 117^T may explain

for the cytotoxic activity exhibited by these microorganisms. As the present of those compounds in *Streptomyces* strains of previous studies were suggested to be responsible for the observed cytotoxic effect on human cancer cell line (5, 75). Overall, majority of the pyrrolopyrazine compounds detected are known to exhibit antioxidant activity. Since antioxidants were suggested to play important role in cellular mechanisms (76), the detection of pyrrolopyrazine compounds in these strains of bacteria could be contributing to the observed cytotoxic effects on cancer cells and neuroprotective effect on SH-SY5Y cells against the insults of H_2O_2.

Taken altogether, the existence of heterocyclic compounds such as phenolics, pyrazines and pyrrolopyrazines as part of the constituents of *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T extracts may account for the observed antibacterial, anticancer activity on cancer cell lines as well as the neuroprotective properties. Based on current findings, rare actinobacteria may serve as important sources for the potential new drugs development.

Table 2: Compounds identified from *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T by using GS-MS.

Extract	No	Retention time (min)	Compound	Formula	Molecular weight (MW)	Quality (%)
MUSC 115^T	1	9.684	methyllaurate	C_8H_{10}	106	80
	2	44.422	2,4-di-tert-butyl phenol	$C_{14}H_{22}O$	206	97
	3	51.592	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	168	90
	4	53.188	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	96
	5	59.008	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	$C_{11}H_{18}N_2O_2$	210	83
	6	70.761	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	$C_{14}H_{16}N_2O_2$	244	76
MUSC 117^T	7	9.936	Butanoic acid, 3-methyl-	$C_5H_{10}O_2$	102	72
	8	10.880	Butanoic acid, 2-methyl-	$C_5H_{10}O_2$	102	53
	9	44.428	2,4-di-tert-butyl phenol	$C_{14}H_{22}O$	206	95
	10	51.563	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	168	90
	11	53.137	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	96
	12	54.865	Methyl n-pentadecanoate	$C_{16}H_{32}O_2$	256	93
	13	59.025	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	$C_{11}H_{18}N_2O_2$	210	64
	14	59.174	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine	$C_{14}H_{22}N_2O_2$	250	53
	15	61.462	Methyl 14-methylhexadecanoate	$C_{18}H_{36}O_2$	284	93
	16	70.749	pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244	92
MUSC 78^T	17	7.538	2-Methylpyrazine	$C_5H_6N_2$	94	80
	18	9.181	Pyrrole, 2-methyl-	C_5H_7N	81	80
	19	13.438	Pyrazine, 2,5-dimethyl-	$C_6H_8N_2$	108	80
	20	17.094	2,3,4-Trithiapentane	$C_2H_6S_3$	126	72
	21	19.383	Pyrazine, 2-ethyl-6-methyl-	$C_7H_{10}N_2$	122	60
	22	19.480	Pyrazine, 2-ethyl-5-methyl-	$C_7H_{10}N_2$	122	95

23	19.555	Pyrazine, trimethyl-	$C_7H_{10}N_2$	122	87
24	24.184	Pyrazine, 3-ethyl-2,5-dimethyl-	$C_8H_{12}N_2$	136	90
25	25.900	4H-Pyran-4-one, 3-hydroxy-2-methyl-	$C_6H_6O_3$	126	70
26	34.935	1H-Indole	C_8H_7N	117	95
27	44.439	2,4-di-tert-butyl phenol	$C_{14}H_{22}O$	206	96
28	45.567	1H-Pyrrole, 2-phenyl-	$C_{10}H_9N$	143	87
29	49.475	1-Naphthalenamine, N-ethyl-	$C_{12}H_{13}N$	171	90
30	50.213	3,4-Dimethyl-2-phenyl-1H-pyrrole	$C_{12}H_{13}N$	171	72
31	51.649	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	168	90
32	53.349	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	97
33	54.596	Methyl 13-methyltetradecanoate	$C_{16}H_{32}O_2$	256	98
34	57.995	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	93
35	59.122	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	95
36	70.743	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	$C_{14}H_{16}N_2O_2$	244	92

4.0 CONCLUSIONS

The results have demonstrated *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78^{T} possessed antibacterial, anticancer and neuroprotective activities. The chemical analysis study afforded a further in depth understanding on the mixture of chemical constituents that present in these strains of bacteria. Based on the literature evidences, the occurrence of these chemical compounds might accounted for the observed bioactivities. In short, the current study has showed these novel rare actinobacteria were able to produce a wide range of bioactive compounds which could serve as potential sources for future drug development. Further in depth studies focusing on isolation and characterization of bioactive principle(s) through bioassay-guided isolation is currently undertaking. As we deeply believe the procedure will eventually enabling us to identify the bioactive principle(s) that present in these mixtures and the findings might potentially generate useful knowledge for the future development of new drug(s).

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Chapter 7

General conclusion, future work and recommendations

7.1 General conclusion

Actinobacteria have been the most productive bacteria for the new drugs development for pharmaceutical industry. Currently, there is much evidence that suggested the novel bioactive compounds with therapeutic application are still waiting to be discovered from these natural sources from new and untapped environment. Therefore, mangrove is a good alternative for the discovery of novel rare actinobacteria and bioactive compounds (Hong *et al.*, 2009). In this study, three rare actinobacteria were discovered from mangrove soils of Tanjung Lumpur, East Coast of Peninsular Malaysia, namely strain MUSC 115^T, MUSC 117^T and MUSC 78^T. Vandamme *et al.* (1996) recommended that new or novel bacteria should be validly named only if their genotypic, chemotaxonomic and phenotypic have been thoroughly and adequately characterized.

The combined methods of polyphasic approach proved that strain MUSC 115^T is a different and new species under the genus *Microbacterium*, therefore it represent a novel species, *Microbacterium mangrovi*. MUSC 115^T is non-spore-forming, Gram-positive and irregular cocci to rods shape and arrangement. It formed yellowish-white colour, circular, raised, smooth and glistening colonies. The phylogenetic analysis showed this novel strain is falls in the genus *Microbacterium.* The 16S rRNA analysis showed that strain MUSC 115^T shared a high degree of sequence similarity with the 16S rRNA from *Microbacterium immunditiarum* SK 18^T (98.1%), *Microbacterium ulmi* XIL02^T (97.8%) and *Microbacterium arborescens* DSM 20754^T (97.5%) while other *Microbacterium* species shared lower similarities, less than 97.5%. Result of polyphasic approach proved that strain MUSC 115^T is a different and new species under the

genus *Microbacterium,* therefore it represent a novel species, *Microbacterium mangrovi* (man.gro'vi. N.L. gen. n. *mangrovi* of a mangrove, the source of the organism).

On the other hand, strain MUSC 117^T showed a range of phenotypic, molecular and chemotaxonomic properties consistent with those of the genus *Sinomonas*. MUSC 117^T is a Gram-positive, non-motile, aerobic, cocci in shape and in irregular arrangement. Colonies are circular, convex, smooth, and form yellowish-white-pigmented on all media tested. The 16S rRNA gene sequences of strain MUSC 117^T formed a distinctive subclade with type strain *S. albida* $LC13^T$ supported by high bootstrap value of 98 % (neigbour-joining algorithm) and 94 % (maximum likelihood algorithm). Thus, strain MUSC 117^T is proposed to be classified as new species named *Sinomonas humi* sp. nov. (hu'mi. L. gen. n. *humi* of soil, ground).

Meanwhile, the 16S rRNA analysis showed that strain MUSC 78^T has closest phylogenetic neighbors with the members of the family *Intrasporangiaceae.* By using polyphasic approach, it was proved that strain MUSC 78^T is different with all the genera under the family *Intrasporangiaceae.* MUSC 78^T is yellowish-white, aerobic, Gram-positive, irregular coccoid to short rod shape, non-motile, and non-spore forming bacteria. The 16S rRNA analysis revealed that strain MUSC 78^T has closest phylogenetic neighbors with the members of the family *Intrasporangiaceae.* The 16S rRNA gene sequences of strain MUSC 78^T showed 98.3% was similar to *Terrabacter lapilli* L-26^T, 98.2% similar to *Intrasporangium oryzae* NRRL B-24470^T and 97.2% similar to *Humibacillus xanthopallidus* KV-663^T. Thus, strain MUSC 78^T represent a novel genus in the family *Intrasporangiaceae,* for which the name *Monashia* gen. nov.

(Mo.na'shi.a. N.L. fem. n. Monashia, a being from Monash University) and the novel species, *Monashia flava* (fla'va. 1. fem. adj. flava, yellow, referring to the colours of the colonies) is proposed

Screening on the bioactivity of three novel strain; *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T proved that these novel strains were able to produce bioactive compound with different biological properties. In this study, the extracts from novel strains able to inhibit the growth of all Gram positive and Gram negative bacteria tested. Furthermore, *Microbacterium mangrovi* MUSC 115^T and *Sinomonas humi* MUSC 117^T extracts were completely inhibiting the growth of *P. aeruginosa* NRBC 112582, *S. typhi* ATCC 19430 and *E. coli* ATCC 25922.

Neuroprotection assay results presented that not all extract able to protect the SHSY5Y neuronal cells against hydrogen peroxides (oxidative stress), streptozotocin (dementia) or cobalt (II) chloride (hypoxia) inducer. Extract form *Microbacterium mangrovi* MUSC 115^T was able to protect the SHSY5Y neuronal cells from the hydrogen peroxides (oxidative stress) inducer. Meanwhile, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T extracts possessed low or no neuroprotective activity on the SHSY5Y neuronal cells against the inducers. In study of cytotoxic effect on human cancer cells, it clearly showed that extracts from *Microbacterium mangrovi* MUSC 115^T and *Monashia flava* MUSC 78^T were possessed strong inhibition of the growth of human cervical cell lines (Ca Ski). However, there were low or no inhibition activities on the growth of human colon cancer cells (HT-29) for all extracts.

In conclusion, the uniqueness of the mangrove such as the water region being alkaline in nature, sediments or soil region having neutral to slightly acidic pH and the environment is rich in carbon and other nutrients provides a unique ecological site to different bacteria (Bhat and Leena, 2013). The data presented in this study conclude that mangrove is an alternative source for discovery and isolation of novel rare actinobacteria, which may produce bioactive compounds with potential therapeutic properties. The 16S rRNA gene sequence used in this study is sufficient to identify the phylogenetic relationship of the novel rare actinobacteria. The discovery of novel rare actinobacteria was accomplished by using the polyphasic approach which combined different methods such as genotypic, chemotaxonomic and phenotypic. The polyphasic approach taken in this study evidenced that this method is able to evaluate, describe and characterize the taxonomy status of new strains. In this study, screening of the bioactive compound provided more understanding into the potential bioactivities possessed by these rare actinobacteria. The results of the screening provide further indication that rare actinobacteria isolated from mangrove environment might be an important source of new bioactive compounds for new drugs development.

7.2 Future works and recommendations

Several approaches can be done in the future as a continuation of this study:

7.2.1 Next-generation sequencing (NGS)

Currently, the standard method to prove the novelty of a bacterial species is the use of DDH and 16S rRNA gene sequencing. However, this combination is only practical for cases in which the 16S rRNA gene sequence similarity between two strains is 97 % (Bull and Koike, 2015). Even though the combine methods are considered as the best approach to characterize novel strain, there is some limitation such as the DDH experiment is labor-intensive, error-prone nature, the cut-off values are not applicable to all genera, lacks reproducibility and cannot be used to establish a comparative reference database incrementally (Tindall *et al.*, 2010). Meanwhile, 16S rRNA is too conserved to differentiate two closely related species (Chun and Rainey, 2014), the presence of nucleotide variations among multiple rRNA operon in single genome and the possibility of the gene being acquired by horizontal gene transfer (HGT) that may affect the relationships between taxa in phylogenetic tree (Ramasamy *et al.*, 2014). Interestingly, this problem can be overcome by comparison of whole genome sequences using next generation sequencing (NGS) technology, which have better resolution in species demarcation and is readily applicable to microbial taxonomy. Some methods based on genome sequences have been proposed to discriminate species including the use of average nucleotide identity (ANI), genome BLAST distance phylogeny (GBDP) and the maximal unique matches index (MUMi) (Kim *et al.*, 2014).

Of these methods, Konstantinidis and Tiedje (2007) suggested that the average nucleotide identity (ANI) was a valid and most widely alternative to DDH in near future for species delineation. It is now generally accepted that ANI values of 95-96% equate to a DDH values of 70% (Goris *et al.*, 2007; Richter and Rosselló-Móra, 2009). To date, ANI methods has been used to describe new subspecies of *Francisella* (Mikalsen *et al.*, 2007), new species of *Burkholderia* (Vanlaere *et al.*, 2009), *Geobacter daltonii* (Prakash *et al.*, 2010), *Vibrio carribeanicus* (Hoffmann *et al.*, 2012), new class of *Dehalococcoides mccartyi* (Löffler *et al.*, 2012), and new genus and species of *Sphaerochaeta; Sphaerochaeta globosa* and *Sphaerochaeta pleomorpha* (Ritalahti *et al.*, 2012). This method is essential for describing new species in large and complex genera, and direct determination of DDH will no longer be needed once the genome sequences for all type strains are achieved in databases. Since genome sequencing includes high-quality of 16S rRNA gene sequence, this will be a direct method at the post-DNA-sequencing phase and genome sequencing also offers an accurate DNA G+C content in the genome (Chun and Rainey, 2014). Moreover, these technologies are no longer restricted to cultivated organisms notably given the insight available from single cell sequencing approaches (Siegl *et al.*, 2011).

The decreasing costs and high throughput of NGS have enabled many genomes to be sequenced. However, there are some drawbacks in bacterial taxonomy; one of the drawbacks is the lack of complete sequenced genomes for many of the major lineages (Klenk and Göker, 2010). Furthermore, the quality of genome sequences available in public databases as the data is either complete or draft sequences (Ricker *et al.*, 2012). The draft genomes in the databases are less informative for taxonomic purposes (Klassen and Currie, 2012). Richter and Rosselló-Móra (2009) and Tindall *et al.* (2010) observed that the genome sequences of species of interest are not

always those of type strains and this represent a major problem when trying to implement genomic data into microbial taxonomy. Ramasamy *et al.* (2014) also stated that some of the genomes sequences from several strains for species with validly published names are currently not available. Ozen *et al.* (2012) argued that result obtained from whole genome method such as ANI results are not consistent with current taxonomy system and should not be used as single tool for bacterial classification. Therefore, different methods must be employed for different levels of taxonomy.

7.2.2 Mechanism of the bioactivity

Although studies regarding bioactivity of genus *Microbacterium* extracts have been studied before, this is the first attempt to study the effectiveness of *Microbacterium,* and also *Sinomonas* and *Monashia* extracts from mangrove environments against pathogenic bacteria, human cancerous cells (colon cancer cells, HT-29 and cervical cancer cells, Ca Ski) and to protect the neuronal cells (SH-SY5Y cells) from the inducers. In particular, screening on the antibacterial showed an interesting result where the extracts were able either to induce cell death (bactericidal) or inhibit cell growth (bacteriostatic). The bioactive compounds in the extracts might be working on several mechanisms such as inhibit DNA synthesis, RNA synthesis, cell wall synthesis or protein synthesis that contribute to the growth inhibition or cell death. Furthermore, the results from cytotoxicity screening can lead to the further investigation on the phase-specific toxicity whether the bioactive compounds in the extracts are toxic to the cancer cells that are actively dividing or in both the proliferating and resting phase. Therefore, these

three species clearly merit further investigation and study in order to identify their mode of action against bacterial pathogens and also their levels of cytotoxicity against normal cells.

7.2.3 Identification and characterization of bioactive compound

Secondary metabolites are rich sources of novel bioactive compounds and drug leads due to their high diversity in chemical structures (Lahlou, 2013). Based on the results of bioactivity screening of the crude extracts, the chemical fingerprint database based on bioactive compounds profiles can be done using gas chromatography-mass spectrometry (GC-MS) (Fiehn *et al.*, 2000), capillary electrophoresis mass spectrometry (CE-MS) (Soga, 2007) or liquid chromatography mass spectrometry (LC-MS) analytical platform (Lu *et al.*, 2014). According to Tang (2011), the study on bioactive compounds profiles or microbial metabolomics will benefits researcher on monitoring and understanding of the interaction between microorganism with its development process and environment since secondary metabolites often result from the interplay between the genotype and the external environment.

7.3 References

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Azman, A.S., Othman, I., Fang, C.M., Chan, K.G., Goh, B.H. & Lee, L.H. (2016). Antibacterial, anticancer and neuroprotective activities of rare actinobacteria from mangrove forest soils. Indian Journal of Microbiology. (Under review)

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Appendices

Microbacterium mangrovi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil

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Strain MUSC 115^T was isolated from mangrove soil of the Tanjung Lumpur river in the state of Pahang, Peninsular Malaysia. Cells of this strain stained Gram-positive and were non-spore-forming, short rods that formed yellowish-white colonies on different agar media. The taxonomy of strain MUSC 115^T was studied by a polyphasic approach, and the organism showed a range of phylogenetic and chemotaxonomic properties consistent with those of the genus *Microbacterium*. The cell-wall peptidoglycan was of type B2β, containing the amino acids ornithine, alanine, glycine, glutamic acid and homoserine. The muramic acid was of the *N*-glycolyl form. The predominant menaquinones detected were MK-12, MK-13 and MK-11. The polar lipids consisted of phosphatidylglycerol, phosphoglycolipid, diphosphatidylglycerol, two unidentified lipids, three unidentified phospholipids and four unidentified glycolipids. The major fatty acids of the cell membrane were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The whole-cell sugars detected were ribose, glucose, mannose and galactose. Based on the 16S rRNA gene sequence, strain MUSC 115^T showed the highest sequence similarity to *Microbacterium immunditiarum* SK 18^T (98.1 %), *M. ulmi* XIL02^T (97.8 %) and *M. arborescens* DSM 20754^T (97.5 %) and lower sequence similarity to strains of other species of the genus *Microbacterium*. DNA–DNA hybridization experiments revealed a low level of DNA–DNA relatedness (less than 24 %) between strain MUSC 115^T and the type strains of closely related species. Furthermore, BOX-PCR fingerprint comparison also indicated that strain MUSC 115^T represented a unique DNA profile. The DNA G+C content determined was 70.9 ± 0.7 mol%, which is lower than that of *M. immunditiarum* SK 18^T. Based on the combination of genotypic and phenotypic data, it is proposed that strain MUSC 115^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium mangrovi* sp. nov. is proposed. The type strain is MUSC 115^T (=MCCC 1K00251^T=DSM 28240^T=NBRC 110089^T).

Members of the genus *Microbacterium*, established by Orla-Jensen (1919), the description of which has been emended by Collins *et al.* (1983), Takeuchi & Hatano (1998) and Krishnamurthi *et al.* (2012), are widespread in nature and have been isolated from diverse habitats such as soil, plants, water, milk products, insects and humans (Collins & Bradbury, 1992; Richert *et al.*, 2007; Dastager *et al.*, 2008; Madhaiyan *et al.*, 2010; Krishnamurthi *et al.*, 2012). At the time of writing, the genus *Microbacterium* contained 84 species with validly published names (http://www.bacterio.net/microbacterium.html). According to Schumann *et al.* (1999), DNA–DNA hybridization assays are vital to the identification of novel species of the genus *Microbacterium*, since the 16S rRNA gene sequence similarity between the closest related type strains is more than 97 % in some

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MUSC 115^T is KF028598.

Three supplementary figures and a supplementary table are available with the online Supplementary Material.

strains and 98–99 % in many strains and can be as high as 99.9 %, as described for the type strains of *Microbacterium oxydans* and *Microbacterium luteolum* (Schumann *et al.*, 1999). Based on the data from a polyphasic approach, we here describe a novel species of the genus *Microbacterium*, *Microbacterium mangrovi* sp. nov., for a bacterium isolated from a mangrove environment in Malaysia.

A study was undertaken to investigate the bacterial diversity in a mangrove forest with the aim of isolating amylolytic strains. A series of bacteria were isolated from mangrove soil samples, one of which produced significant starch hydrolysis activity and was chosen for further study. Polyphasic analysis of this strain, MUSC 115^T, suggested that it belonged to the genus *Microbacterium* (Takeuchi & Hatano, 1998). Strain MUSC 115^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located in the mangrove forest of Tanjung Lumpur in the state of Pahang, Peninsular Malaysia, in December 2012. Topsoil samples of the upper 20 cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored in −20 °C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C; Takahashi *et al.*, 1996). Five grams of the pretreated air-dried soil was mixed with 45 ml sterilized water and mill-ground, spread onto a selective isolation medium, starch casein agar (SCA; Küster & Williams, 1964) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and then incubated at 28 °C for 7 days. Pure cultures of strain MUSC 115^T were maintained on ISP2 medium at 28 °C and as glycerol suspensions (20 %, v/v) at −20 °C.

Cultural characteristics of strain MUSC 115^T were determined following growth on ISP2 and ISP7 media (Shirling & Gottlieb, 1966), SCA (Küster & Williams 1964), *Streptomyces* agar (SA; Atlas 1993), actinomycetes isolation agar (AIA; Atlas 1993) and nutrient agar (NA; MacFaddin, 2000) for 7 days at 28 °C. Light microscopy (80*i*; Nikon) and scanning electron microscopy (JEOL-JSM 6400) were used to observe the morphology of the strain after incubation on ISP2 medium at 28 °C for 7 days. The ISCC-NBS colour charts (Kelly, 1964) were used to determine the names and designations of colony colours. Gram staining was performed by the standard Gram reaction and was confirmed by using KOH lysis (Cerny, 1978). Growth was tested at 4–52 °C at intervals of 4 °C on ISP2 medium. The pH range for growth was tested between pH 4.0 and 10.0 at intervals of 1 pH unit. NaCl tolerance was tested using tryptic soy broth (TSB) and salt concentrations ranging from 0 to 18 % (w/v) at intervals of 2 %. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Production of melanoid pigments was examined using tyrosine agar (ISP7 medium). Haemolytic activity was assessed in blood agar medium containing 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) human blood (Carrillo *et al.*, 1996). Plates were examined for haemolysis after incubation at 32 °C for 7 days. Lipase, amylase, cellulase, chitinase, protease and xylanase activities were determined by growing cells on ISP2 medium following the protocols described by Meena *et al.* (2013). The presence of a clear zone around colonies signifies the potential of isolates for surfactant production. Antibiotic susceptibility tests were performed by the disc diffusion method as described by Shieh *et al.* (2003). *Microbacterium immunditiarum* JCM 14034^T, *M. ulmi* JCM 14282^T and *M. arborescens* DSM 20754^T were obtained from the JCM and DSMZ as indicated and tested concurrently with strain MUSC 115^T. Antimicrobials used and the amounts per disc (Oxoid) were as follows: ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), nalidixic acid (30 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). Cells were resistant to nalidixic acid and sensitive to other antimicrobials tested. Carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions. Differential physiological characteristics between strain MUSC 115^T and the type strains of closely related species of the genus *Microbacterium* are described in the species description and in Table 1.

Genomic DNA extraction for PCR was performed as described by Hong *et al.* (2009). The 16S rRNA gene was amplified using the universal primers p27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′). PCR amplification and purification of the product were completed as described by Hong *et al.* (2009). The 16S rRNA gene sequence of strain MUSC 115^T was aligned with sequences of closely related type strains that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL_X software (Thompson *et al.*, 1997). The alignment was verified manually and adjusted prior to the reconstruction of the phylogenetic tree using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms with the MEGA version 5.2 software (Tamura *et al.*, 2011). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) was used for calculations of sequence similarity. The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980).

The nearly complete 16S rRNA gene sequence was established for strain MUSC 115^T (1484 bp) and phylogenetic trees were reconstructed to determine the phylogenetic position of this strain (Fig. 1 and Fig. S1, available in the online Supplementary Material). Phylogenetic analysis exhibited that strain MUSC 115^T was closely related to *M. immunditiarum* SK 18^T, as they formed a distinct clade supported by a high bootstrap value (86 %), indicating a high confidence level of this association

Table 1. Differentiation of strain MUSC 115^T and the type strains of closely related species of the genus *Microbacterium*

Strains: 1, MUSC 115^T; 2, *M. immunditiarum* JCM 14034^T; 3, *M. ulmi* JCM 14282^T; 4, *M. arborescens* DSM 20754^T. Data were obtained in this study. +, Positive; −, negative; (+), weakly positive. All strains were positive for growth in 1 % NaCl and utilization of dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, lactose, melibiose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, 3-methyl glucose, inosine, D-sorbitol, D-mannitol, glycerol, gelatin, glycyl L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-serine, pectin, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, *p*-hydroxyphenylacetic acid, D-lactic acid methyl ester, L-lactic acid, L-malic acid, Tween 40, γ-aminobutyric acid, α-hydroxybutyric acid, α-ketobutyric acid, acetoacetic acid, propionic acid and acetic acid.

Characteristic	1	2	3	4
Major fatty acids (%)				
anteiso-$C_{15:0}$	38.0	22.0	35.1	42.9
anteiso-$C_{17:0}$	31.2	39.2	30.0	24.9
iso-$C_{16:0}$	8.6	19.2	16.7	28.5
Colony colour on:*				
ISP2 agar	YWH	CY	WH	DO
SA	YWH	POY	PWH	MRO
NA	YWH	PYWH	PWH	MOY
Luria–Bertani agar	YWH	PYWH	PWH	MOY
Growth at/in:				
16 °C	+	+[a]†	+[b]	+
32 °C	+	+[a]	+[b]	−[c]
40 °C	(+)	(+)	−[b]	−[c]
pH 5	+	−[a]	+[b]	−
pH 9	+	−[a]	−[b]	−
4 % NaCl	+	−	+	+
8 % NaCl	−	−	+[b]	+
Catalase	+	+[a]	−[b]	+[c]
Haemolytic activity	−	−	−	+
Hydrolysis of:				
Starch (amylase)	+	−	+[b]	−[c]
CM-cellulose	−	+	+[b]	+
Casein	−	−[a]	+[b]	−
Xylan	−	−[a]	+[b]	−
Carbon source utilization				
Stachyose	+	−	+	+
Raffinose	+	−	+	+
N-Acetyl-β-D-mannosamine	+	−	+	+
N-Acetyl-D-galactosamine	+	−	+	−
D-Galactose	−	+	+	+
D-Fucose	−	+	+	+
L-Fucose	−	+	+	+
L-Rhamnose	−	+	+	+
D-Arabitol	+	−	+	+
myo-Inositol	+	−	+	+
D-Glucose 6-phosphate	−	+	+	+
D-Fructose 6-phosphate	−	+	+	+
D-Aspartic acid	+	−	+	−
D-Serine	−	+	+	−
L-Pyroglutamic acid	+	−	+	+
Glucuronamide	−	+	+	+
Mucic acid	+	−	+	−
Quinic acid	+	−	+	−
D-Saccharic acid	+	−	+	−
Methyl pyruvate	+	−	+	+
α-Ketoglutaric acid	−	+	+	+
D-Malic acid	+	−	+	+
Formic acid	+	−	+	−
Chemical sensitivity assays				
Troleandomycin	−	+	+	+
Lincomycin	−	+	+	+
Tetrazolium violet	−	+	+	+
Tetrazolium blue	−	+	+	+
Lithium chloride	+	−	+	+
Sodium butyrate	+	−	+	+
Sodium bromate	−	+	+	+

*CY, Creamish yellow; DO, dirty orange; MOY, moderate orange–yellow; MRO, moderate reddish orange; POY, pale orange–yellow; PWH, pale white; PYWH, pale yellowish white; WH, white; YWH, yellowish white.

†Result in accordance with that published for: *a*, *M. immunditiarum* SK 18^T by Krishnamurthi *et al.* (2012); *b*, *M. ulmi* XIL02^T by Rivas *et al.* (2004); *c*, *M. arborescens* DSM 20754^T by Takeuchi & Hatano (1998).

(Fig. 1). This association was also supported in the phylogenetic tree reconstructed using the maximum-likelihood algorithm (Fig. S1). 16S rRNA gene sequence analysis of strain MUSC 115^T showed the highest similarity to *M. immunditiarum* SK 18^T (98.1 %), followed by *M. ulmi* XIL02^T (97.8 %) and *M. arborescens* DSM 20754^T (97.5 %); sequences similarities of less than 97.5 % were obtained to the type strains of other species of the genus *Microbacterium*. According to Stackebrandt & Goebel (1994), a 16S rRNA gene sequence divergence of more than 3 % is generally accepted as a criterion for delineating different species. The sequence divergence between MUSC 115^T and the most closely related type strain, *M. immunditiarum* SK 18^T, was 1.9 %, a value that indicated a strong possibility of strain MUSC 115^T representing a novel species of the genus *Microbacterium*.

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies were obtained after growth in TSB at 28 °C for 7 days on a rotary shaker. DNA–DNA hybridization analysis of strain MUSC 115^T and closely related type strains was performed by the Identification Service of the DSMZ. Extraction of genomic DNA from strain MUSC 115^T, *M. immunditiarum* JCM 14034^T, *M. ulmi* JCM 14282^T and *M. arborescens* DSM 20754^T was performed as described by Cashion *et al.* (1977). DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a

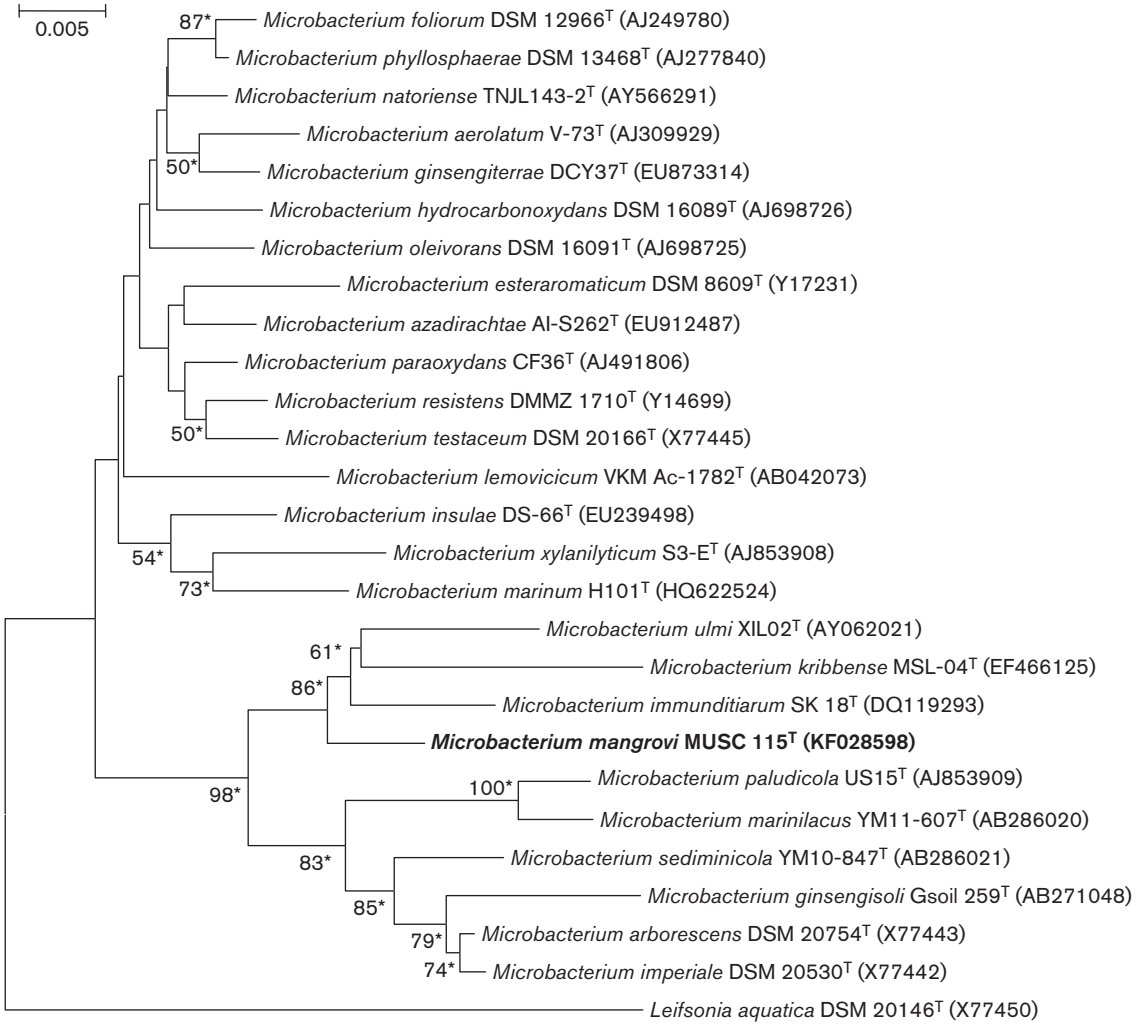

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on 16S rRNA gene sequences showing relationships between strain MUSC115^T and representatives of related taxa. Bootstrap values (>50 %) based on 1000 resampled datasets are shown at branch nodes. Bar, 5 substitutions per 1000 nucleotide positions. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood algorithm. *Leifsonia aquatica* DSM 20146^T was used as an outgroup.

model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). The DNA–DNA relatedness between strain MUSC 115^T and *M. immunditiarum* JCM 14034^T (23.6 ± 0.5 %), *M. ulmi* JCM 14282^T (26.2 ± 2.7 %) and *M. arborescens* DSM 20754^T (16.3 ± 1.1 %) was significantly lower than 70 %, the recommended threshold value for the delineation of genomic species (Wayne *et al.* 1987). These results suggested that strain MUSC 115^T did not belong to any of these species. Moreover BOX-PCR fingerprint analysis was used to compare strain MUSC 115^T and the reference strains using the primer BOX-A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic *et al.*, 1991). BOX-PCR cycling parameters were 5 min at 94 °C for pre-denaturation, 35 cycles each of 30 s at 94 °C for denaturation, 30 s at 53 °C for annealing and 7 min at 65 °C for extension and a final extension at 65 °C for 8 min. The PCR products were visualized by 2 % agarose gel electrophoresis. The rep-PCR results are shown in Fig. S2. It is evident that strain MUSC 115^T yielded a unique BOX-PCR fingerprint compared with closely related type strains. These results are in agreement with the results of DNA–DNA hybridization, that strain MUSC 115^T represents a novel species.

Cellular fatty acid analysis of strain MUSC 115^T and closely related type strains was carried out by the Identification Service of the DSMZ. Cell mass of strain MUSC 115^T and the reference strains was harvested from TSB after incubation at 28 °C for 5 days. The fatty acids were extracted and prepared according to the standard protocol of the MIDI System (Sasser, 1990). The complete fatty acid profiles of strain MUSC 115^T and the reference strains are

shown in Table S1. The predominant cellular fatty acids in strain MUSC 115^T were anteiso-$C_{15:0}$ (38.0 %) and anteiso-$C_{17:0}$ (31.2 %). Other fatty acids detected were iso-$C_{16:0}$ (8.6 %), iso-$C_{18:0}$ (6.7 %), iso-$C_{17:0}$ (5.6 %) and trace amounts of $C_{18:0}$, iso-$C_{15:0}$ and anteiso-$C_{19:0}$ (Table S1). The fatty acid profile of strain MUSC 115^T was similar to those of the reference strains, which also contained the predominant fatty acids anteiso-$C_{15:0}$ (22.0–42.9 %) and anteiso-$C_{17:0}$ (24.9–39.2 %) (Table S1). Strain MUSC 115^T contained a fatty acid profile that was quantitatively different from that of *M. immunditiarum* JCM 14034^T, particularly in the amounts of anteiso-$C_{15:0}$ and iso-$C_{16:0}$ (Tables 1 and S1). These fatty acids were present in almost equal amounts (22.0 and 19.2 %) in *M. immunditiarum* JCM 14034^T whereas, for strain MUSC 115^T, the amount of anteiso-$C_{15:0}$ (38.0 %) was nearly double that found in *M. immunditiarum* JCM 14034^T.

Analysis of the peptidoglycan amino-acid composition and whole-cell sugars of strain MUSC 115^T was carried out by Identification Service of the DSMZ. The analyses were carried out according to Schumann (2011). The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan of strain MUSC 115^T contained the amino acids ornithine (Orn), alanine, glycine, glutamic acid and homoserine (Hse). The molar ratio was 1.0 Orn : 0.7 Hse : 1.4 Gly : 0.5 Ala : 1.0 Glu. 3-*threo*-hydroxyglutamic acid could not be detected. The identity of these amino acids was confirmed by agreement in the GC retention time with those of authentic standards and by the detection of characteristic MS fragment ions of their derivatives. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C) of the peptidoglycan contained the peptides Orn–D-Ala, Gly–Orn and Gly–Glu. From these analytical data, it was concluded that strain MUSC 115^T contained the peptidoglycan type B2β (Schleifer & Kandler, 1972). The *N*-acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida & Aida (1977). The hydrolysate (0.5 M H_2SO_4, 2 h, 100 °C) of cells of strain MUSC 115^T contained ribose, glucose, mannose and galactose. Galactose has been detected in many other species of the genus *Microbacterium* (Rivas *et al.*, 2004; Krishnamurthi *et al.*, 2012). The detection of ribose, glucose and mannose was noteworthy, as these sugars were not found in the closely related type strains *M. immunditiarum* JCM 14034^T and *M. ulmi* JCM 14282^T, exhibiting a significant difference between strain MUSC 115^T and these type strains.

Analysis of respiratory quinones and polar lipids was carried out by the Identification Service of the DSMZ. The cellular polar lipids were extracted and analysed by TLC (Kates, 1986). The predominant menaquinones detected were MK-12 (62 %), MK-13 (28 %) and MK-11 (8 %). A minor amount of MK-10 (1 %) was also detected. The polar lipids consisted of phosphatidylglycerol, phosphoglycolipid, diphosphatidylglycerol, two unidentified lipids, three unidentified phospholipids and four unidentified glycolipids (Fig. S3a). Comparison of the polar lipid profiles of strain MUSC 115^T and the closest related type strain *M. immunditiarum* JCM 14034^T (Fig. S3b) indicated that they shared four types of polar lipid, namely unidentified lipids and glycolipids, phosphatidylglycerol and diphosphatidylglycerol. However, differences in the polar lipid profiles, e.g. strain MUSC 115^T contained phospholipid and phosphoglycolipid, which were lacking in *M. immunditiarum* JCM 14034^T, indicated that strain MUSC 115^T was distinct.

Genomic DNA for the determination of G+C content was extracted according to Cashion *et al.* (1977) and the G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). The DNA G+C content of strain MUSC 115^T was 70.9±0.7 mol%, within the range of 69.0–76.6 mol% described for species within the genus *Microbacterium* (Takeuchi & Hatano, 1998; Rivas *et al.*, 2004; Krishnamurthi *et al.*, 2012). On the basis of phenotypic, chemotaxonomic, DNA–DNA hybridization and phylogenetic analyses, strain MUSC 115^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium mangrovi* sp. nov. is proposed.

Description of *Microbacterium mangrovi* sp. nov.

Microbacterium mangrovi (man.gro′vi. N.L. gen. n. *mangrovi* of a mangrove).

Cells stain Gram-positive and are non-spore-forming, irregular cocci or rods. Cells form yellowish-white colonies on all agar media tested. Good growth is observed on ISP2 medium, NA and SA after 7 days at 28 °C; cells grow moderately well on Luria–Bertani agar, whereas cells grow poorly on SCA, ISP7 medium and AIA. Using TSB, the ranges of NaCl concentration, temperature and pH for growth are 0–4 % (w/v) NaCl, 15–36 °C and pH 5.0–8.0; optimal growth occurs at 0–2 % NaCl, 28–32 °C and pH 5.0–8.0. Cells are positive for catalase but negative for haemolytic activity. Soluble starch is hydrolysed, but CM-cellulose, chitin, tributyrin (lipase), casein and xylan are not. The following compounds are utilized as sole carbon sources: dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, lactose, melibiose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, α-D-glucose, D-mannose, D-fructose, 3-methyl glucose, inosine, D-sorbitol, D-mannitol, D-arabitol, *myo*-inositol, glycerol, D-aspartic acid, gelatin, glycyl L-proline, pectin, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxyphenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, Tween 40, γ-aminobutyric acid, α-hydroxybutyric acid, β-hydroxy-DL-butyric acid, α-ketobutyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid. The following compounds are not utilized as sole carbon sources: D-galactose, D- and L-fucose, L-rhamnose, D-glucose 6-phosphate, D-fructose 6-phosphate, D-serine, L-galactonic acid lactone, glucuronamide, citric acid, α-ketoglutaric acid and bromosuccinic acid. L-Alanine, L-arginine, L-aspartic acid, L-glutamic acid,

L-histidine, L-pyroglutamic acid and L-serine are utilized as sole nitrogen sources. In chemical sensitivity assays, cells are sensitive towards fusidic acid, D-serine, troleandomycin, minocycline, lincomycin, guanine hydrochloride, Niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue and sodium bromate, while cells are resistant to 1 % sodium lactate, rifamycin RV, nalidixic acid, lithium chloride, potassium tellurite, aztreonam and sodium butyrate. Cells are sensitive to (per disc) ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). Resistant to nalidixic acid (30 μg). The peptidoglycan is of type B2β, containing the amino acids ornithine, alanine, glycine, glutamic acid and homoserine. The acyl type is *N*-glycolyl. The predominant menaquinones are MK-12, MK-13 and MK-11. The major fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The whole-cell sugars are ribose, glucose, mannose and galactose. The polar lipids consist of phosphatidylglycerol, phosphoglycolipid, diphosphatidylglycerol, two unidentified lipids, three unidentified phospholipids and four unidentified glycolipids.

The type strain is MUSC 115^T (=MCCC 1K00251^T=DSM 28240^T=NBRC 110089^T), isolated from mangrove soil collected from the Tanjung Lumpur mangrove forest in the state of Pahang, Peninsular Malaysia. The G+C content of the genomic DNA of the type strain is 70.9±0.7 mol%.

Acknowledgements

This work was supported by a University of Malaya for High Impact Research grant (UM-MOHE HIR Nature Microbiome grant no. H-50001-A000027) awarded to C. K.-G and an External Industry Grant (vote no. GBA-808138) awarded to L. L.-H. The authors are grateful to researchers from the DSMZ for performing the chemotaxonomic analyses and to Professor Jean Euzéby for support in providing the Latin etymology of the new name.

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International Journal of Systematic and Evolutionary Microbiology (2015), **65**, 996–1002 **DOI** 10.1099/ijs.0.000053

Sinomonas humi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil

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Strain MUSC 117^T was isolated from mangrove soil of the Tanjung Lumpur forest in Pahang, Malaysia. This bacterium was yellowish-white pigmented, Gram-staining-positive, rod–coccus shaped and non-motile. On the basis of 16S rRNA gene sequence, strain MUSC 117^T exhibited highest sequence similarity to *Sinomonas atrocyanea* DSM 20127^T (98.0 %), *Sinomonas albida* LC13^T (97.9 %) and *Sinomonas soli* CW 59^T (97.8 %), and lower (<97.6 %) sequence similarity to other species of the genus *Sinomonas*. DNA–DNA hybridization experiments revealed a low level of DNA–DNA relatedness (less than 27 %) between strain MUSC 117^T and closely related species. Chemotaxonomically, the peptidoglycan type was A3α, containing the amino acids lysine, serine, glycine, alanine, glutamic acid and muramic acid. The whole-cell sugars detected were rhamnose, ribose, glucose, galactose and a smaller amount of mannose. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and five unidentified glycolipids. The major fatty acids (>10.0 %) of the cell membrane were anteiso-$C_{15:0}$ (39.4 %), $C_{18:1}\omega7c$ (17.7 %), anteiso-$C_{17:0}$ (17.2 %) and iso-$C_{16:0}$ (11.4 %). The predominant respiratory quinones detected were MK-9(H_2) and MK-9. The DNA G+C content was 67.3 mol%. A comparison of BOX-PCR fingerprints indicated that strain MUSC 117^T represented a unique DNA profile. Results based on a polyphasic approach showed that strain MUSC 117^T represents a novel species of the genus *Sinomonas*, for which the name *Sinomonas humi* sp. nov. is proposed. The type strain of *Sinomonas humi* sp. nov. is MUSC 117^T (=DSM 29362^T=MCCC 1K00410^T=NBRC 110653^T).

The genus *Sinomonas* was established by Zhou *et al.* (2009) with the description of the strain *Sinomonas flava* CW 108^T and the reclassification of *Arthrobacter atrocyaneus* as *Sinomonas atrocyanea* (type strain DSM 20127^T) (Kuhn & Starr, 1960; Zhou *et al.*, 2009). Following the publication of the genus *Sinomonas*, the description of *Sinomonas soli* and reclassification of *Arthrobacter echigonensis* and *Arthrobacter albidus* (Ding *et al.*, 2009) as *Sinomonas echigonensis* and *Sinomonas albida* occurred (Zhou *et al.*, 2012). At the time of writing, the genus *Sinomonas* comprised seven species (http://www.bacterio.net/): the type species *S. flava* and *S. atrocyanea* (Zhou *et al.*, 2009), *S. albida*, *S. echigonensis*, *S. soli*, '*Sinomonas notoginsengisoli*' and '*Sinomonas mesophila*' (Zhou *et al.*, 2012; Prabhu *et al.*, 2014; Zhang *et al.*, 2014). Members of these species were isolated from habitats such as polluted forest soil and filtration substrate (Ding *et al.*, 2009; Zhou *et al.*, 2009, 2012). DNA–DNA hybridization assays were vital for the identification of novel species in the genus *Sinomonas* since the 16S rRNA gene sequence similarity between closely related type strains is more than 98 % for strains *S. albida* LC13^T and *S. echigonensis* LC 10^T, and more than 99.4 % for *S. atrocyanea* DSM 20127^T, *S. flava* CW 108^T and *S. soli* CW 59^T. On the basis of data from a polyphasic approach, we describe a novel species of the genus *Sinomonas*, *Sinomonas humi* sp. nov., an actinobacterial isolate from the mangrove environment in Malaysia.

A study was undertaken to investigate the bacterial diversity in mangrove forest with the aim of isolating

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MUSC 117^T is KC964537.

Two supplementary figures are available with the online Supplementary Material.

amylolytic strains. A series of bacteria were isolated from a mangrove soil sample, one of which produced significant starch hydrolysis activity and was chosen for further study. The polyphasic analysis of this strain, MUSC 117^T, suggested that it belonged to the genus *Sinomonas* (Zhou *et al.*, 2009, 2012). Strain MUSC 117^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located in the mangrove forest of Tanjung Lumpur in the state of Pahang, Peninsular of Malaysia, in December 2012. Topsoil samples of the upper 20 cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored at −20 °C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C; Takahashi *et al.*, 1996). Five grams of the pretreated air-dried soil was mixed with 45 ml sterilized water and mill ground, spread onto isolation medium: starch casein agar (SCA; Küster & Williams, 1964) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), then incubated at 28 °C for 7 days. Pure cultures of strain MUSC 117^T were maintained on ISP2 medium (Shirling & Gottlieb, 1966) at 28 °C and as glycerol suspensions (20 %, v/v) at −20 °C.

Cultural characteristics of strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T, *S. soli* KCTC 19389^T and *S. flava* JCM 16034^T were determined following growth on ISP2 and ISP7 media (Shirling & Gottlieb, 1966), SCA, Streptomyces agar (SA; Atlas, 1993), Actinomycetes isolation agar (AIA; Atlas, 1993) and nutrient agar (MacFaddin, 2000) for 7 days at 28 °C. The Inter-Society Color Council–National Bureau of Standards (ISCC-NBS) colour charts (Kelly, 1964) were used to determine the names and designations of the colony colours. Light microscopy (80*i*; Nikon) and scanning electron microscopy (JSM-6400; JEOL) were used to observe the morphology of the strain after incubation on ISP2 medium at 28 °C for 7 days. Gram staining was performed by using the standard Gram reaction and was confirmed by using KOH lysis (Cerny, 1978). Growth was tested at 10–44 °C at intervals of 4 °C on ISP2 medium. The pH range for growth was tested between pH 4.0 and 10.0 at intervals of 1 pH unit. NaCl tolerance was tested using tryptic soy broth (casein, 17 g; soybean meal, 3 g; glucose, 2.5 g; dipotassium hydrogen phosphate, 2.5 g; distilled water, 1 l; pH 7.3) and NaCl concentrations ranging from 0–18 % (w/v) at intervals of 2 %. Haemolytic activity was assessed in blood agar medium containing 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) human blood (Carrillo *et al.*, 1996). Plates were examined for haemolysis after incubation at 32 °C for 7 days. Amylase, cellulase, chitinase, lipase, protease and xylanase activities were determined by growing cells on ISP2 medium following protocols as described by Meena *et al.* (2013). Presence of a clear zone around colonies signifies the potential of isolates for surfactant production. Catalase activity and production of melanoid pigments were determined following protocols described by Lee *et al.* (2014a). Antibiotic susceptibility tests were performed by using the disc diffusion method as described by Shieh *et al.* (2003). Antimicrobials used and the amounts per disc (Oxoid) were as follows: ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), nalidixic acid (30 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). Carbon source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions. All of the physiological assays mentioned were tested concurrently for strains MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T. These type strains were obtained from NBRC, JCM and KCTC as indicated. Differential physiological characteristics between strain MUSC 117^T and the type strains of closely related species of the genus *Sinomonas* are described in the species description and in Table 1.

Genomic DNA extraction for PCR was performed as described by Hong *et al.* (2009). PCR amplification of the 16S rRNA gene was completed as described by Lee *et al.* (2014b). The 16S rRNA gene sequence of strain MUSC 117^T was aligned with sequences of closely related type strains that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL X software (Thompson *et al.*, 1997). The alignment was verified manually and adjusted prior to the reconstruction of phylogenetic trees using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms with MEGA version 5.2 software (Tamura *et al.*, 2011). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) was used for calculations of sequence similarity. The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980).

The almost complete 16S rRNA gene sequence was established for strain MUSC 117^T (1493 bp) and phylogenetic trees were reconstructed to decide the phylogenetic position of this strain (Figs 1 and 2). Phylogenetic analysis showed that strain MUSC 117^T was closely related to all of the type strains of species of the genus *Sinomonas* as they formed a distinct clade supported by a high bootstrap value (100 %), indicating the highest confidence level of this association (Fig. 1). Moreover strain MUSC 117^T formed a distinctive subclade with type strain *S. albida* LC13^T supported by high bootstrap values of 99 % (Fig. 1) and 97 % (Fig. 2). Analysis of the 16S rRNA gene sequence of strain MUSC 117^T showed that this isolate had highest similarity to the type strain *S. atrocyanea* DSM 20127^T (98.0 %), followed by *S. albida* LC13^T (97.9 %) and *S. soli* CW 59^T (97.8 %), whereas sequence similarities of less than 97.6 % were obtained to other species of the genus *Sinomonas*. DNA–DNA hybridization has been vital in the identification of novel species in the genus *Sinomonas* as the 16S rRNA gene sequence similarities between type

Table 1. Differentiation of strain MUSC 117^T and type strains of closely related species of the genus *Sinomonas*

Strains: 1, *Sinomonas humi* sp. nov. MUSC 117^T; 2, *S. atrocyanea* NBRC 12956^T; 3, *S. albida* JCM 21830^T; 4, *S. soli* KCTC 19389^T; 5, *S. flava* JCM 16034^T. Data from all strains were obtained from this study. +, Positive; −, negative; (+), weakly positive. All strains were amylolytic and positive for growth at 40 °C and assimilation of dextrin, maltose, gentiobiose, turanose, raffinose, α-lactose, melibiose, methyl β-D-glucoside, D-galactose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-arabitol, *myo*-inositol, glycerol, D-fructose 6-phosphate, L-glutamic acid, L-histidine, L-galactonic acid lactone, citric acid, D-malic acid, L-malic acid, γ-aminobutyric acid, α-hydroxybutyric acid, acetic acid and formic acid. All strains were negative for assimilation of D-mannose, L-pyroglutamic and L-serine.

Characteristic	1	2	3	4	5
Colony colour (on AIA)*	YW	YW	YW	POY	PY
Growth at:					
10 °C	(+)	(+)	+†	(+)	(+)
pH 8	+	(+)	(+)†	+‡	+§
4 % NaCl	(+)	+	(+)	−‡	−§
Catalase	+	+‡	+†	+‡	(+)§
Carbon source utilization					
Cellobiose	+	+	+	−	+
Sucrose	+	+	+†	−	+§
Stachyose	+	+	+	−	+
D-Salicin	−	+	+	+	−
α-D-Glucose	+	+	−	−	−
D-Fructose	−	+	−	−	−
D-Fucose	+	−	+	+	+
D-Mannitol	−	−	−	+	+
D-Glucose 6-phosphate	−	+	+	−	+
D-Aspartic acid	+	−	+	+	+
L-Alanine	−	+	−	−	+
Gelatin	−	−	+	−	+
Pectin	−	−	−	+	−
D-Galacturonic acid	−	−	+	+	+
Mucic acid	−	+	+	−	+
α Ketoglutaric acid	+	−	+	+	+
Bromosuccinic acid	−	+	+	−	−
β-Hydroxy-DL-butyric acid	−	+	+	−	+
Acetoacetic acid	−	+	−	−	+
L-lactic acid	−	−	+	+	−
Propionic acid	−	−	+	+	−
Chemical sensitivity assays					
D-Serine	−	+	+	−	+
Nalidixic acid	+	−	+	−	+
Lithium chloride	+	−	+	−	+
Potassium tellurite	−	+	+	−	+

*YW, yellowish white; POY, pale orange–yellow; PY, pale yellow.
†Results in accordance with those published for *S. albida* JCM 21830^T by Ding *et al.* (2009).
‡Results in accordance with those published for *S. atrocyanea* NBRC 12956^T and *S. soli* KCTC 19389^T by Zhou *et al.* (2012).
§Results in accordance with those published for *S. flava* JCM 16034^T by Zhou *et al.* (2009).

strains are >98 % for some strains, and 99.5 % as described for *S. atrocyanea* DSM 20127^T and *S. soli* CW 59^T.

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies were obtained after growing in tryptic soy broth at 28 °C for 7 days on a rotary shaker. Genomic DNA extraction and DNA–DNA hybridization of strain MUSC 117^T and closely related type strains were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Genomic DNA extraction from strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T was performed as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). The DNA–DNA relatedness values between strain MUSC 117^T and *S. atrocyanea* NBRC 12956^T (19.8 ± 4.2 %), *S. albida* JCM 21830^T (26.8 ± 1.8 %) and *S. soli* KCTC 19389^T (20.8 ± 2.7 %) were significantly lower than 70 %, the threshold value for the delineation of genomic species (Wayne *et al.*, 1987). These results supported the notion that strain MUSC 117^T represents a novel species. Rep-PCR fingerprint analysis was used to compared strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T, *S. soli* KCTC 19389^T and *S. flava* JCM 16034^T using the primer BOX-A1R (5′-CTACGGCAAGG-CGACGCTGACG-3′) (Versalovic *et al.*, 1991; Lee *et al.*, 2014c). The PCRs were performed in a final volume of 50 µl according to protocol of SolGent 2 × *Taq* Plus PCR Smart mix using a Kyratec PCR Supercycler with cycling conditions as described by Lee *et al.* (2014d). PCR products were visualized by 2 % agarose gel electrophoresis. The rep-PCR results showed that strain MUSC 117^T exhibited a unique BOX-PCR fingerprint compared with closely related type strains (Fig. S1, available in the online Supplementary Material), therefore supporting the results of DNA–DNA hybridization that strain MUSC 117^T represents a novel species.

Analysis of the peptidoglycan amino acid composition and whole-cell sugars of strain MUSC 117^T was carried out by Identification Service of the DSMZ. The analyses were carried out according to Schumann (2011). The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan of strain MUSC 117^T contained the amino acids lysine (Lys), serine (Ser), glycine (Gly), alanine (Ala), glutamic acid (Glu) and muramic acid (Mur). The molar ratio was 1.0 Lys : 0.9 Ser : 0.7 Gly : 2.5 Ala : 1.8 Glu : 0.9 Mur. The identity of these amino acids was confirmed by agreement of the GC retention time with those of authentic standards and by characteristic mass spectrometric fragment ions of the derivatives. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C,) of the peptidoglycan contained the peptides L-Ala-D-Glu, D-Glu-Gly, L-Ala-D-Ala, L-Ser-L-Ala, L-Lys-L-Ser and L-Lys-D-Ala. From these analytical data, it was concluded that strain MUSC 117^T contained the peptidoglycan type

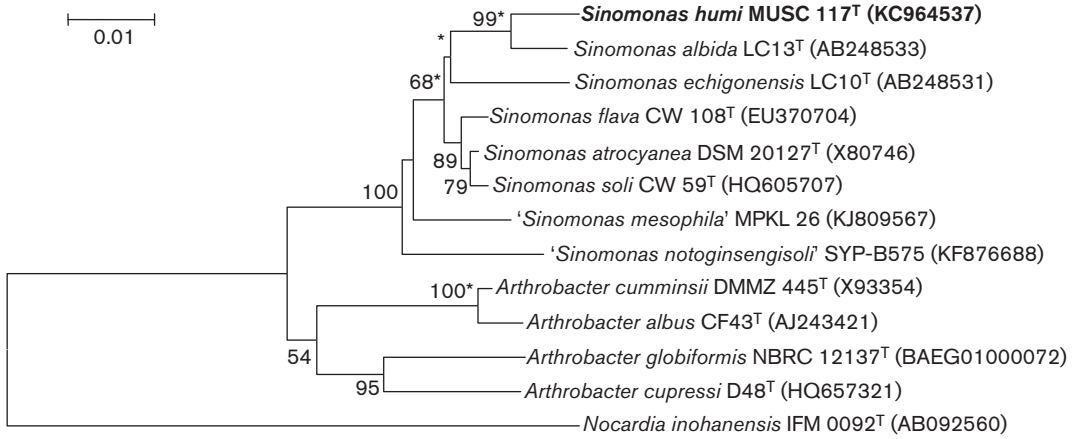

Fig. 1. Phylogenetic tree calculated using the neighbour-joining algorithm indicating the phylogenetic relationship of strain MUSC 117^T to members of species of the genus *Sinomonas*. There were a total of 1334 positions in the final dataset. Bootstrap values (>50 %) based on 1000 resampled datasets are shown at branch nodes. Bar, 10 substitutions per 1000 nucleotide positions. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm.

A3α (Schleifer & Kandler, 1972), which is consistent with the peptidoglycan type reported for type strains of species of the genus *Sinomonas* (Schleifer & Kandler, 1972; Koch *et al.*, 1995; Zhou *et al.*, 2009, 2012). The whole-cell sugars detected for strain MUSC 117^T were rhamnose, ribose, glucose, galactose and a smaller amount of mannose. Strain MUSC 117^T contained sugars such as ribose, galactose and rhamnose, which were detected in other species of the genus *Sinomonas* (Zhou *et al.*, 2009, 2012). The detection of rhamnose was noteworthy, as this sugar was not found in other species of the genus *Sinomonas*; this demonstrated a significant difference between strain MUSC 117^T and these type strains.

Cellular fatty acids analyses of strain MUSC 117^T, *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T were carried out by the Identification Service of the DSMZ. The cell mass of strain MUSC 117^T and related type strains was harvested from tryptic soy broth after incubation at 28 °C for 2 days. The fatty acids were extracted and prepared according to the standard protocol of the MIDI (Microbial Identification) system (Sasser,

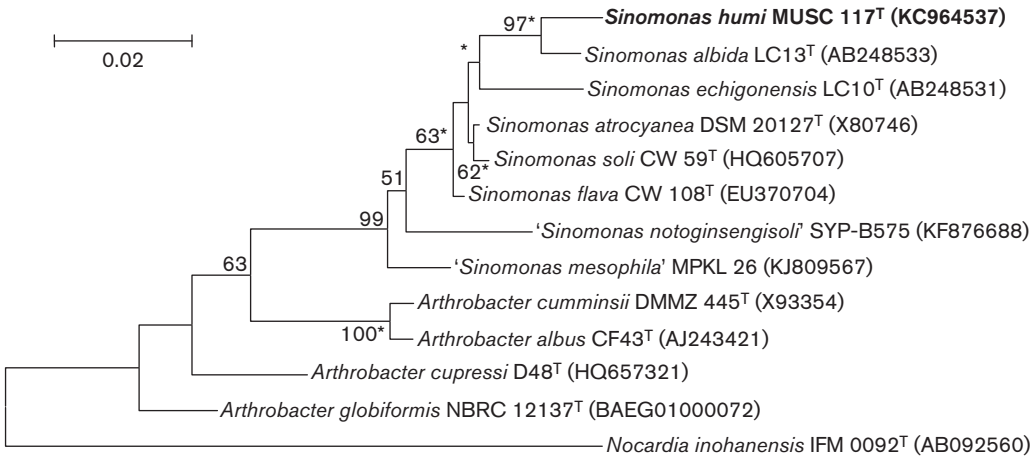

Fig. 2. Phylogenetic tree calculated using the maximum-likelihood algorithm indicating the phylogenetic relationship of strain MUSC 117^T to members of species of the genus *Sinomonas*. There were a total of 1334 positions in the final dataset. Bootstrap values (>50 %) based on 1000 resampled datasets are shown at branch nodes. Bar, 20 substitutions per 1000 nuceotide positions. Asterisks indicate that the corresponding nodes were also recovered using the neighbour-joining tree-making algorithm.

1990). The complete fatty acids profiles of strain MUSC 117^T and closely related type strains are shown in Table 2. The major cellular fatty acids were anteiso-$C_{15:0}$ (39.4 %), $C_{18:1}\omega7c$ (17.7 %), anteiso-$C_{17:0}$ (17.2 %), iso-$C_{16:0}$ (11.4 %) and iso-$C_{15:0}$ (9.8 %). Other fatty acids detected were iso-$C_{17:0}$ (1.6 %), $C_{16:0}$ (1.5 %), and trace amounts of iso-$C_{14:0}$, $C_{14:0}$ and $C_{15:0}$ (Table 2). The fatty acids profile of MUSC 117^T was consistent with those of closely related type strains such as *S. atrocyanea* NBRC 12956^T, *S. albida* JCM 21830^T and *S. soli* KCTC 19389^T, which contained fatty acids anteiso-$C_{15:0}$ (39.4–57.4 %), anteiso-$C_{17:0}$ (8.8–22.1 %) and iso-$C_{16:0}$ (4.1–11.4 %) as their major fatty acids. However, strain MUSC 117^T contained a fatty acid, $C_{18:1}\omega7c$ (17.7 %), that was not detected in any of the related type strains (Table 2).

Analysis of respiratory quinones and polar lipids was carried out by the Identification Service of the DSMZ. The cellular polar lipids were extracted and analysed by TLC (Kates, 1986). The predominant menaquinones detected were MK-9(H_2) (86 %) and MK-9 (9 %). Minor amounts of MK-10(H_2) (1 %) and MK-8 (<1 %) were also detected. This menaquinone composition is in agreement with the report of Zhou *et al.* (2012) that the predominant menaquinone of members of the genus *Sinomonas* is MK-9(H_2). The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and five unidentified glycolipids (Fig. S2). These polar lipids are commonly found in the type strains of species of the genus *Sinomonas* (Zhou *et al.*, 2009, 2012).

Genomic DNA of strain MUSC 117^T for the determination of G+C content was extracted according to the protocol of Cashion *et al.* (1977). The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). The DNA G+C content of strain MUSC 117^T was 67.3 mol%; this is within the range of 66.9–71.8 mol% reported for species of genus *Sinomonas* (Zhou *et al.*, 2012). On the basis of phenotypic, DNA–DNA hybridization, chemotaxonomic and phylogenetic analyses, strain MUSC 117^T merits assignment to a novel species of the genus *Sinomonas*, for which the name *Sinomonas humi* sp. nov. is proposed.

Table 2. Cellular fatty acid composition of strain MUSC 117^T and closely related species of the genus *Sinomonas*

Strains: 1, *Sinomonas humi* sp. nov. MUSC 117^T; 2, *S. atrocyanea* NBRC 12956; 3, *S. albida* JCM 21830^T; 4, *S. soli* KCTC 19389^T. Data were determined in this study and represent percentages of total fatty acids. –, <0.1 % or not detected.

Fatty acid	1	2	3	4
iso-$C_{13:0}$	–	–	–	0.2
anteiso-$C_{13:0}$	–	0.1	–	0.2
iso-$C_{14:0}$	0.9	2.4	0.5	2.8
$C_{14:0}$	0.3	0.5	0.2	0.7
iso-$C_{15:0}$	9.8	11.7	17.4	19.9
anteiso-$C_{15:0}$	39.4	57.4	51.9	56.4
$C_{15:0}$	0.2	0.3	–	0.3
iso-$C_{16:0}$	11.4	10.0	4.1	7.8
$C_{16:0}$	1.5	1.9	0.7	1.8
iso-$C_{17:0}$	1.6	1.1	3.1	1.3
anteiso-$C_{17:0}$	17.2	14.7	22.1	8.8
$C_{18:1}\omega7c$	17.7	–	–	–

Description of *Sinomonas humi* sp. nov.

Sinomonas humi (hu′mi. L. gen. n. *humi* of soil, ground).

Cells stain Gram-positive and are strictly aerobic, non-motile and bent rods. Colonies are circular, convex and form yellowish-white-pigmented colonies on all different media tested. Good growth is observed on ISP2 medium, AIA, SA and nutrient agar after 7 days at 28 °C; cells grow moderately on SCA, while cells grow poorly on ISP7 medium. Using tryptic soy broth, the NaCl tolerance, temperature and pH ranges for growth are 0–4 % (w/v), 24–40 °C, pH 5.0–8.0, respectively, and optimal growth occurs at 0–2 % NaCl, 28–36 °C and pH 6–7. Cells are positive for catalase and amylase activity, but negative for cellulase, chitinase, xylanase, protease, lipase and haemolytic activities. The utilization of the following compounds as sole carbon sources can be used for differentiation from closely related type species of the genus *Sinomonas*: cellobiose, sucrose, stachyose, α-D-glucose, D-fucose, D-fructose 6-phosphate, D-aspartic acid and α-ketoglutaric acid. The following compounds are not utilized as sole carbon sources: D-salicin, D-mannitol, D-glucose 6-phosphate, gelatin, pectin, D-galacturonic acid, mucic acid, L-lactic acid, bromosuccinic acid, β-hydroxy-DL-butyric acid, acetoacetic acid and propionic acid. Sole nitrogen sources such as L-alanine are not utilized. In chemical sensitivity assays, cells are sensitive to D-serine and potassium tellurite; resistant to nalidixic acid and lithium chloride. Cells are sensitive to ampicillin, ampicillin sulbactam, cefotaxime, cefuroxime, cephalosporin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline and vancomycin. The peptidoglycan is of type A3α, containing the amino acids lysine, serine, glycine, alanine, glutamic acid and muramic acid. The whole-cell sugars are rhamnose, ribose, glucose, galactose and a smaller amount of mannose. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and five unidentified glycolipids. The major fatty acids are anteiso-$C_{15:0}$, $C_{18:1}\omega7c$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The predominant respiratory quinones are MK-9(H_2) and MK-9.

The type strain is MUSC 117^T (=DSM 29362^T=MCCC 1K00410^T=NBRC 110653^T), isolated from mangrove soil collected from the state of Pahang in Peninsular Malaysia. The genomic DNA G+C content of the type strain is 67.3 mol%.

Acknowledgements

This work was supported by a University of Malaya for High Impact Research Grant (UM-MOHE HIR Nature Microbiome Grant no. H-50001-A000027) awarded to K.-G. C., Fundamental Research Grant Scheme (FRGS/1/2013/SKK01/MUSM/03/3) and External Industry

Grant (Biotek Abadi/GBA-808813) awarded to L.-H. L. The authors are thankful to Professor Jean Euzéby for support in the Latin etymology of the novel species names.

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Monashia flava gen. nov., sp. nov., an actinobacterium of the family *Intrasporangiaceae*

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A novel actinobacterial strain, MUSC 78^T, was isolated from a mangrove soil collected from Peninsular Malaysia. The taxonomic status of this strain was determined using a polyphasic approach. Comparative 16S rRNA gene sequence analysis revealed that strain MUSC 78^T represented a novel lineage within the class *Actinobacteria*. Strain MUSC 78^T formed a distinct clade in the family *Intrasporangiaceae* and was related most closely to members of the genera *Terrabacter* (98.3– 96.8 % 16S rRNA gene sequence similarity), *Intrasporangium* (98.2–96.8 %), *Humibacillus* (97.2 %), *Janibacter* (97.0–95.3 %), *Terracoccus* (96.8 %), *Kribbia* (96.6 %), *Phycicoccus* (96.2–94.7 %), *Knoellia* (96.1–94.8 %), *Tetrasphaera* (96.0–94.9 %) and *Lapillicoccus* (95.9 %). Cells were irregular rod-shaped or cocci and stained Gram-positive. The cell-wall peptidoglycan type was A3γ, with LL-diaminopimelic acid as the diagnostic diamino acid. The main cell-wall sugar was mannose and lower amounts of galactose and rhamnose were present. The predominant menaquinone was MK-8(H_4). The polar lipid profile consisted of phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol and phosphoglycolipid. The predominant fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$.The DNA G+C content was 73.1 mol%. Based on this polyphasic study, MUSC 78^T exhibited phylogenetic and phenotypic differences from members of the genera of the family *Intrasporangiaceae*, and therefore a novel species of a new genus, *Monashia flava* gen. nov., sp. nov., is proposed. The type strain of *Monashia flava* is MUSC 78^T (=DSM 29621^T=MCCC 1K00454^T=NBRC 110749^T).

The family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2000) was redescribed by Zhi *et al.* (2009) and the family contains an array of actinobacteria, in addition to the type genus *Intrasporangium* (Kalakoutskii *et al.*, 1967), and has MK-8(H_4) or MK-8 as the major menaquinones and LL-diaminopimelic acid (LL-DAP), *meso*-DAP or L-ornithine (L-Orn) in the cell-wall peptidoglycan (Martin *et al.*, 1997; Maszenan *et al.*, 2000; Groth *et al.*, 2001, 2002; Hanada *et al.*, 2002; Kageyama *et al.*, 2005, 2007, 2008a, b; Jung *et al.*, 2006; Lee & Lee, 2007). At the time of writing, the family *Intrasporangiaceae* contained 19 genera, including 16 genera summarized by Zhi *et al.* (2009) and the recently described *Marihabitans* (Kageyama *et al.*, 2008b), *Fodinibacter* (Wang *et al.*, 2009) and *Ornithinibacter* (Xiao *et al.*, 2011). It is possible to distinguish these genera from each other on the basis of phenotypic and genotypic characteristics. The present investigation was designed to determine the taxonomic status of a novel actinobacterial strain, MUSC 78^T, that contained LL-DAP in the cell-wall peptidoglycan and MK-8(H_4) as the predominant menaquinone. To determine the taxonomic position of strain MUSC 78^T, a polyphasic approach was used to determine the phylogenetic, chemotaxonomic and phenotypic characteristics of the novel strain. The results indicated that strain MUSC 78^T represents a novel species of a new genus, for which the name *Monashia flavus* gen. nov., sp. nov. is proposed.

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain MUSC 78^T is KF682157.

Two supplementary figures are available with the online Supplementary Material.

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Strain MUSC 78^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located at the mangrove forests of the Tanjung Lumpur in the state of Pahang, Peninsular Malaysia, in December 2012. Samples of the upper 20 cm topsoil layer (after removing the top 2–3 cm) were collected using an aseptic metal trowel, placed in sterile plastic bags and stored in −20 °C. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C; Takahashi *et al.*, 1996). Five grams of air-dried soil was mixed with 45 ml sterilized water and ground using a mill and then the suspension was spread onto a selective isolation medium, starch casein agar (SCA; Küster & Williams, 1964) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and incubated at 28 °C for 7 days. Isolate MUSC 78^T was maintained on R2A agar medium at 28 °C and as glycerol suspensions (20 %, v/v) at −20 °C.

Cultural characteristics of strain MUSC 78^T were determined following growth on ISP 2 and ISP 7 media (Shirling & Gottlieb, 1966), SCA, *Streptomyces* agar (SA; Atlas 1993), *Actinomycetes* isolation agar (AIA; Atlas 1993) and nutrient agar (MacFaddin, 2000) for 7 days at 28 °C. The ISCC-NBS colour charts were used to determine the names and designations of colony colours (Kelly, 1964). Light microscopy (80i; Nikon) and scanning electron microscopy (JEOL-JSM 6400) were used to observe the morphologies of strains after incubation on R2A agar medium at 28 °C for 7 days. Gram staining was done following the standard Gram reaction and was confirmed by using KOH lysis (Cerny, 1978). Growth was tested at 4–44 °C at intervals of 4 °C on R2A agar medium. NaCl tolerance was tested using trypticase soy broth (TSB) and salt concentrations of 0–14 % (w/v) at intervals of 2 %. Growth was tested between pH 4.0 and 10.0 at intervals of 1 pH unit. The production of melanoid pigments and catalase activity were assessed following the protocols described by Lee *et al.* (2014a). Haemolytic activity was assessed on blood agar medium containing 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) horse blood (Carrillo *et al.*, 1996). Plates were examined for haemolysis after incubation at 32 °C for 7 days. Lipase, amylase, cellulase, chitinase, protease and xylanase activities were determined by growing cells on R2A agar medium and following the protocols described by Meena *et al.* (2013). The presence of clear zones around colonies signifies the potential of isolates for surfactant production. Antibiotic susceptibility tests were performed by the disc diffusion method as described by Shieh *et al.* (2003). Antimicrobials (Oxoid) used were as follows: ampicillin, ampicillin sulbactam, cefotaxime, cefuroxime, cephalosporin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, penicillin G, streptomycin, tetracycline and vancomycin. Cells were resistant to nalidixic acid but sensitive to the other antimicrobials used. Carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions (Biolog).

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies was obtained after growing in TSB at 28 °C for 5 days on a rotary shaker. Analysis of peptidoglycan amino-acid composition and sugars was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The analyses were performed according to published protocols (Schumann, 2011). Major diagnostic sugars of strain MUSC 78^T were obtained following the procedure described by Whiton *et al.* (1985) and analysed by TLC on cellulose plates according to Staneck & Roberts (1974). Analysis of respiratory menaquinones and polar lipids was carried out by the Identification Service of the DSMZ. The cellular polar lipids were extracted and analysed by TLC (Kates, 1986). Cellular fatty acid analyses of strain MUSC 78^T were carried out by the Identification Service of the DSMZ. Cell mass of strain MUSC 78^T was harvested from TSB after incubation at 28 °C for 5 days. The fatty acids were extracted and prepared according to the standard protocol of the MIDI Microbial Identification system (Sasser, 1990).

Genomic DNA extractions were carried out as described by Hong *et al.* (2009). PCR amplification of the 16S rRNA gene and sequencing of the purified products were done as described by Lee *et al.* (2014b). The 16S rRNA gene sequence of strain MUSC 78^T was aligned with sequences of closely related type genera classified in the family *Intrasporangiaceae* that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL X software (Thompson *et al.*, 1997). The alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic tree. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms with MEGA version 6.0 (Tamura *et al.*, 2013). Calculations of levels of sequence similarity were carried out using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980). The genomic DNA of strain MUSC 78^T for the determination of G+C content was extracted according to Cashion *et al.* (1977). The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989).

Strain MUSC 78^T formed Gram-stain-positive, non-motile, aerobic, non-spore-forming cocci or irregular rod-shaped cells (Fig. 1). Cells formed yellowish-white-pigmented colonies on ISP 2 medium and AIA. Good growth was observed on R2A agar medium, ISP 2 medium, ISP 7 medium, Luria–Bertani agar, nutrient agar and SA after 7 days at 28 °C, while cells grew moderately on AIA and SCA. Cells were positive for catalase and haemolytic activities. Strain MUSC 78^T was positive for hydrolysis of chitin, soluble starch, casein and CM-cellulose, but negative for hydrolysis of tributyrin (lipase) and xylan. Using TSB (without NaCl), the NaCl tolerance, temperature and pH ranges for growth were 0–4 %, 24–36 °C and pH 6.0–8.0

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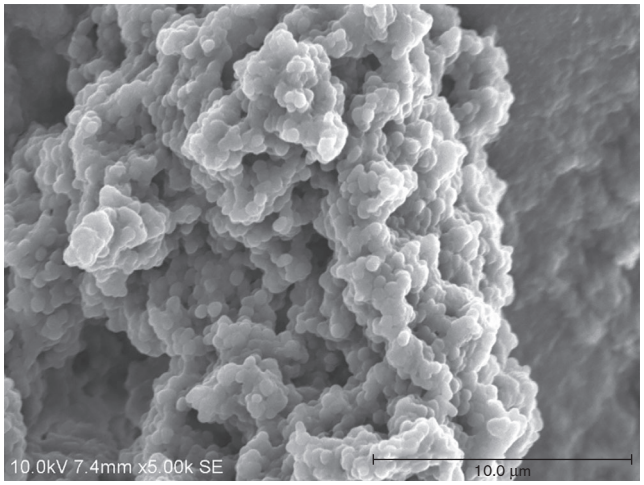

Fig. 1. Scanning electron micrograph of cells from a 5 day old culture of strain MUSC 78^T grown at 28 °C on R2A agar medium. Bar, 10 µm.

and optimal growth occurred at 0–2 % NaCl, 28–32 °C and pH 7.0. The morphological, cultural and physiological properties of strain MUSC 78^T are given in the genus and species descriptions. The organism could be distinguished from members of the family *Intrasporangiaceae* based on several chemotaxonomic characteristics (Table 1).

The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan of strain MUSC 78^T contained LL-DAP, glycine (Gly), glutamic acid (Glu), alanine (Ala) and muramic acid (Mur). The molar ratio was 1.2 LL-DAP/3.0 Gly/1.0 Glu/1.5 Ala/0.9 Mur. The identity of these amino acids was confirmed by agreement in the GC retention time with those of authentic standards and by characteristic MS fragment ions of the derivatives. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C) of the peptidoglycan contained the peptides L-Ala–D-Glu, Gly–D-Glu, Gly–D-Ala, Gly3, LL-DAP–D-Ala and LL-DAP–Gly. These data indicated that strain MUSC 78^T contains the peptidoglycan type A3γ (Schleifer & Kandler, 1972). The cell-wall sugars of strain MUSC 78^T were mannose and (at lower amounts) galactose and rhamnose. The menaquinones consisted of MK-8(H_4) (80 %), MK-8 (1 %) and MK-8(H_2) (1 %). The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphoglycolipid (Fig. S1, available in the online Supplementary Material). The major fatty acids of strain MUSC 78^T were iso-$C_{15:0}$ (34.8 %), anteiso-$C_{15:0}$ (16.7 %), iso-$C_{16:0}$ (14.5 %), iso-$C_{14:0}$ (8.6 %), anteiso-$C_{17:0}$ (4.6 %), $C_{16:0}$ (2.7 %) and $C_{18:0}$ (2.6 %) (Table 1).

The DNA G+C content of strain MUSC 78^T was 73. 1 mol%, a result that is within the range (68–74.1 %) described for members within the family *Intrasporangiaceae* (Table 1).

The nearly complete 16S rRNA gene sequence was determined for strain MUSC 78^T (1488 bp). Phylogenetic trees were reconstructed based on the 16S rRNA gene sequences (Figs 2 and S2). Comparative 16S rRNA gene sequence analysis showed that strain MUSC 78^T falls within the evolutionary radiation occupied by the family *Intrasporangiaceae* (Figs 2 and S2). The closest phylogenetic neighbours were members of the genera in the family *Intrasporangiaceae* (suborder *Micrococcineae*). Strain MUSC 78^T showed highest 16S rRNA gene sequence similarity to *Terrabacter lapilli* LR-26^T (98.3 %), *Intrasporangium oryzae* NRRL B-24470^T (98.2 %), *Humibacillus xanthopallidus* KV-663^T (97.2 %), *Janibacter anophelis* CCUG 49715^T (97.0 %), *Terracoccus luteus* DSM 44267^T (96.8 %), *Kribbia dieselivorans* N113^T (96.6 %), *Phycicoccus cremeus* V2M29^T (96.2 %), *Knoellia sinensis* DSM 12331^T (96.1 %), *Tetrasphaera japonica* ACM 5116^T (96.0 %) and *Lapillicoccus jejuensis* R-Ac013^T (95.9 %). Strain MUSC 78^T showed the closest evolutionary distance to the type strains of members of the genus *Terrabacter* at a low nucleotide sequence similarity (98.3–96.8 %). However, strain MUSC 78^T could be differentiated from the genus *Terrabacter* by the presence of anteiso-$C_{15:0}$ as a major fatty acid and the presence of phosphatidylglycerol. In addition, the genera *Intrasporangium*, *Humibacillus* and *Terracoccus*, which contain LL-DAP as the major diagnostic diamino acid in the peptidoglycan, do not contain phosphoglycolipid as a diagnostic phospholipid (Table 1). Of the other phylogenetically closely related neighbours, members of the genera *Janibacter*, *Kribbia*, *Phycicoccus*, *Knoellia*, *Tetrasphaera*, *Fodinibacter* and *Oryzihumus* are clearly different from strain MUSC 78^T as these genera contain *meso*-DAP as the diagnostic diamino acid (Table 1). Other genera such as *Ornithinibacter*, *Ornithinicoccus*, *Ornithinimicrobium* and *Serinicoccus* are different from strain MUSC 78^T as they contain L-Orn as the diagnostic diamino acid (Table 1). Differential chemotaxonomic characteristics between strain MUSC 78^T and other genera belonging to the family *Intrasporangiaceae* are summarized in Table 1. Therefore, on the basis of phylogenetic data and differential chemotaxonomic characteristics, strain MUSC 78^T does not belong to any existing genera in the family *Intrasporangiaceae*.

Based on its distinct phylogenetic position within the family *Intrasporangiaceae*, together with characteristic cell morphology, and chemotaxonomic and physiological properties, strain MUSC 78^T should be classified as representing a novel species in a new genus of the family *Intrasporangiaceae*, for which the name *Monashia flavus* gen. nov., sp. nov. is proposed.

Description of *Monashia* gen. nov.

Monashia gen. nov. (Mo.na′shi.a. N.L. fem. n. *Monashia* from Monash University).

Aerobic, non-motile, non-spore-forming, Gram-stain-positive actinobacterium of irregular coccoid to short rod-shaped cells. The predominant menaquinone is MK-8(H_4). The polar lipids are phosphatidylglycerol,

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Table 1. Differential characteristics between strain MUSC 78^T and genera of the family *Intrasporangiaceae*

Taxa: 1, strain MUSC 78^T; 2, *Terrabacter* (data from Collins *et al.*, 1989, unless otherwise indicated); 3, *Intrasporangium* (Schumann *et al.*, 1997); 4, *Humibacillus* (Kageyama *et al.*, 2008a); 5, *Janibacter* (Martin *et al.*, 1997, unless otherwise indicated); 6, *Terracoccus* (Prauser *et al.*, 1997); 7, *Kribbia* (Jung *et al.*, 2006); 8, *Phycicoccus* (Lee, 2006); 9, *Knoellia* (Groth *et al.*, 2002); 10, *Tetrasphaera* (Maszenan *et al.*, 2000); 11, *Lapillicoccus* (Lee & Lee, 2007); 12, *Arsenicicoccus* (Collins *et al.*, 2004); 13, *Ornithinibacter* (Xiao *et al.*, 2011), 14, *Ornithinicoccus* (Groth *et al.*, 1999); 15, *Ornithinimicrobium* (Groth *et al.*, 2001); 16; *Serinicoccus* (Yi *et al.*, 2004); 17, *Fodinibacter* (Wang *et al.*, 2009); 18, *Marihabitans* (Kageyama *et al.*, 2008b); 19, *Oryzihumus* (Kageyama *et al.*, 2005). ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Cell morphology	Irregular rods to cocci	Irregular rods	Hyphae	Irregular rods	Coccoid to rod-shaped	Cocci	Irregular short rods or cocci	Cocci	Coccoid to rod-shaped	Cocci	Cocci	Cocci	Branching hyphal forms	Cocci	Irregular and cocci	Cocci	Irregular short rods	Irregular short rods and cocci	Irregular rods
Wall diamino acid	LL-DAP	LL-DAP	LL-DAP	LL-DAP	*meso*-DAP	LL-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	LL-DAP	LL-DAP	L-Orn	L-Orn	L-Orn	L-Orn	*meso*-DAP	*meso*-DAP	*meso*-DAP
DNA G+C content (mol%)	73.1	71–73	68	69–70	70	73	69–70	74	68–69	68–71	74.1	72	69.6	72	70–71	72	72	70	72–73
Major menaquinones(s)	MK-8(H_4)	MK-8(H_4)	MK-8	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(H_4)
Major fatty acids*	i-$C_{15:0}$, ai-$C_{15:0}$, i-$C_{16:0}$	i-$C_{15:0}$, i-$C_{14:0}$, i-$C_{16:0}$†	ai-$C_{15:0}$, i-$C_{16:0}$	i-$C_{15:0}$, iso-$C_{16:0}$, i-$C_{14:0}$, $C_{17:0}\omega8c$	i-$C_{16:0}$, $C_{17:1}\omega8c$, $C_{18:1}\omega9c$, $C_{17:0}$‡	i-$C_{15:0}$, ai-$C_{15:0}$, $C_{16:0}$	10-Me $C_{18:0}$, i-$C_{16:0}$, $C_{18:1}\omega9c$, $C_{16:0}$, $C_{18:0}$	$C_{17:1}\omega8c$, i-$C_{16:0}$, i-$C_{15:0}$	i-$C_{15:0}$, i-$C_{17:0}$, i-$C_{16:0}$, ai-$C_{17:0}$	i-$C_{16:0}$, ai-$C_{17:0}$, i-$C_{16:1}$, i-$C_{15:0}$, $C_{16:1}$, i-$C_{14:0}$, i-$C_{16:0}$	i-$C_{16:0}$, $C_{17:1}\omega8c$, i-$C_{15:0}$	i-$C_{16:0}$, $C_{18:1}$, i-$C_{15:0}$, ai-$C_{15:0}$	i-$C_{18:1}\omega9c$, i-$C_{16:0}$, i-$C_{15:0}$, $C_{17:0}$	i-$C_{15:0}$, ai-$C_{15:0}$, i-$C_{17:1}$, $C_{15:0}$	i-$C_{16:0}$, i-$C_{15:0}$, ai-$C_{15:0}$	i-$C_{15:0}$, i-$C_{16:0}$, i-$C_{17:1}$, ai-$C_{15:0}$, ai-$C_{17:0}$	$C_{18:1}\omega9c$, $C_{16:0}$	i-$C_{17:0}$, $C_{17:1}\omega8c$, $C_{18:1}\omega9c$	i-$C_{16:0}$, i-$C_{15:0}$, i-$C_{14:0}$
Polar lipid(s)§	DPG, PE, PG, PGL, PI	DPG, PE, PI, GL	ND	PE	DPG, PG, PI	DPG, PG, PI, PL	ND	DPG, PE, PI	DPG, PG, PE	DPG, PG, PI, APL, PL	ND	ND	DPG, PG, PE, PI, GL	DPG, PG, PI, PS, PL	DPG, PG, PI, PL, GL	DPG, PG, PI, GL	DPG, PE, PG, PI, 2PL	DPG, PG, PL, PIMs	DPG, PG, PGL, PL

*Major fatty acids are defined as constituting >10 % of the total fatty acid content: i, iso ; ai, anteiso; Me, methyl.

†Data from Montero-Barrientos *et al.* (2005).

‡Data from Kämpfer *et al.* (2006).

§APL, unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; PL, unknown phospholipid; PS, phosphatidylserine; PGL, unknown phosphoglycolipid; GL, unknown glycolipid.

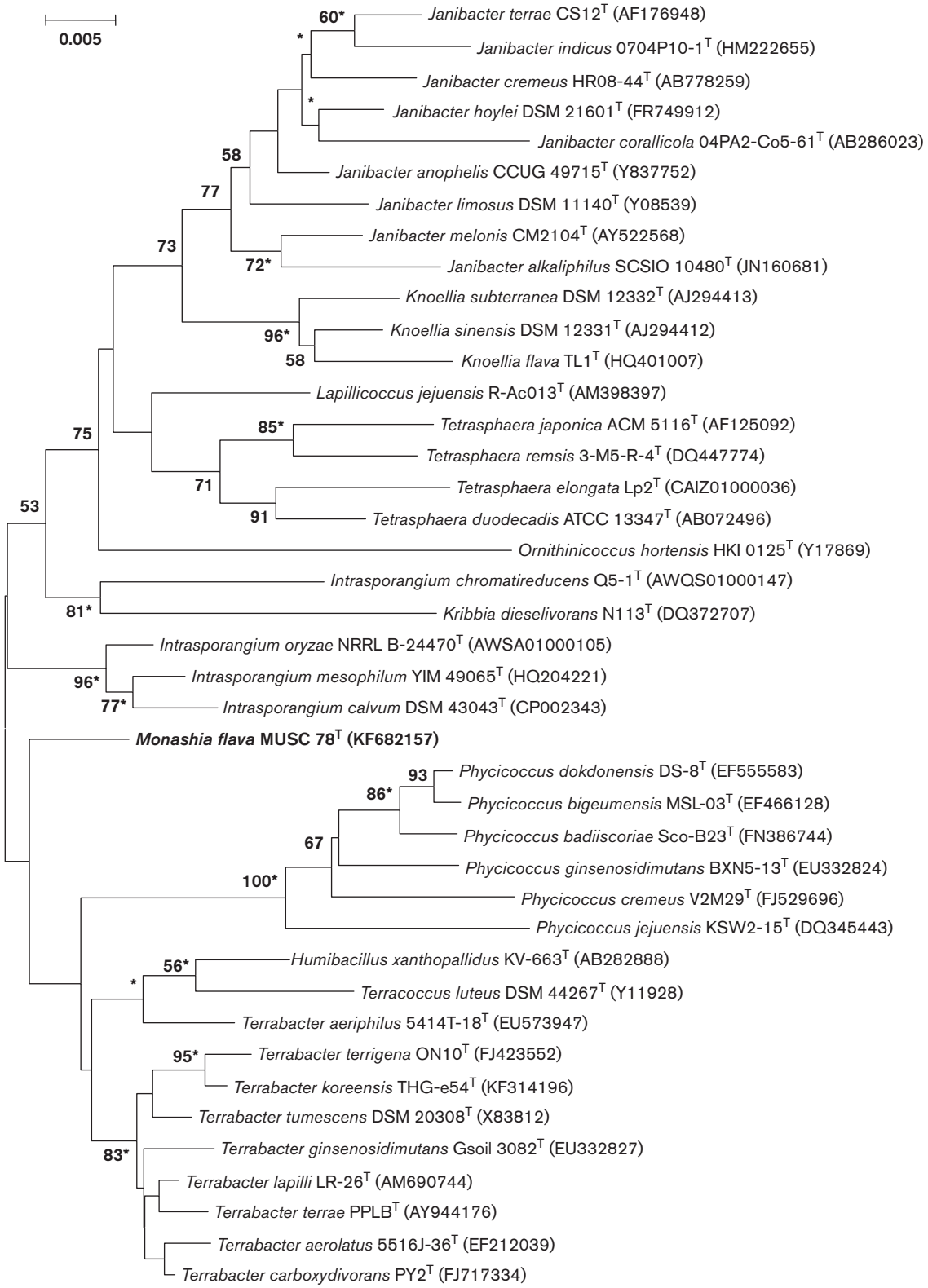

Fig. 2. Phylogenetic neighbour-joining tree (Saitou & Nei, 1987) based on 16S rRNA gene sequences of strain MUSC 78^T and representatives of the family *Intrasporangiaceae*. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm. Bootstrap values (based on 1000 replicates) are shown as percentages at each node for values above 50 %. Bar, 0.005 substitutions per nucleotide position.

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phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol and phosphoglycolipid. The major cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The peptidoglycan contains LL-DAP as the diagnostic diamino acid and the peptidoglycan type is A3γ. The cell-wall sugars are mannose and (at lower amounts) galactose and rhamnose.

Description of *Monashia flava* sp. nov.

Monashia flava (fla′va. l. fem. adj. *flava* yellow, referring to the colour of the colonies).

Has the following properties in addition to those given for the genus. Cells form yellowish-white-pigmented colonies on ISP 2 medium and AIA. Good growth is observed on R2A agar medium, ISP 2 medium, ISP 7 medium, Luria-Bertani agar, nutrient agar and SA after 7 days at 28 °C, while cells grow moderately well on AIA and SCA. Using TSB (without NaCl), the NaCl tolerance, temperature and pH ranges for growth are 0–4 %, 24–36 °C and pH 6.0–8.0 and optimal growth occurs at 0–2 % NaCl, 28–32 °C and pH 7.0. Cells are positive for catalase and haemolytic activities. Positive for hydrolysis of chitin, soluble starch, casein and CM-cellulose, but negative for hydrolysis of tributyrin (lipase) and xylan. The following compounds are utilized as sole carbon sources: dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, α-lactose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-arabitol, D-glucose 6-phosphate, D-fructose 6-phosphate, gelatin, glycyl L-proline, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxyphenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, citric acid, D-malic acid, L-malic acid, bromosuccinic acid, Tween 40, γ-aminobutyric acid, α-hydroxybutyric acid, α-ketobutyric acid, acetoacetic acid, acetic acid and formic acid. The following compounds are not utilized as sole carbon sources: melibiose, 3-methyl glucose, D-fucose, D-mannitol, *myo*-inositol, glycerol, D-aspartic acid, D-serine, pectin, L-galactonic acid lactone, L-lactic acid, α-ketoglutaric acid, β-hydroxy-DL-butyric acid and propionic acid. Sole nitrogen sources such as L-alanine, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid and L-serine are utilized. L-Glutamic acid is not utilized as sole nitrogen source. In chemical sensitivity assays, cells are resistant towards chemicals including fusidic acid, D-serine, troleandomycin, minocycline, lincomycin, guanine HCl, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue and sodium bromate, 1 % sodium lactate, rifamycin RV, nalidixic acid, lithium chloride, potassium tellurite, aztreonam and sodium butyrate.

The type strain is MUSC 78^T (=DSM 29621^T=MCCC 1K00454^T=NBRC 110749^T), which was isolated from mangrove soil collected from the Kuantan, the city of Pahang State in Peninsular Malaysia. The G+C content of the genomic DNA of the type strain is 73.1 mol%.

Acknowledgements

This work was supported by the High Impact Research Grant from the University of Malaya (UM-MOHE HIR Nature Microbiome Grant No. H-50001-A000027) to C. K.-G. and External Industry Grant (Biotek Abadi - Vote No. GBA-808138), Fundamental Research Grant Scheme (FRGS/1/2013/SKK01/MUSM/03/3), MOSTI Science-Fund Grant (Project No. 06-02-10-SF0300) awarded to L.-H. L. We thank Drs Peter Schumann, Susanne Verbarg and Brian Tindall from DSMZ for performing the chemotaxonomic analyses. We are grateful to Professor Dr Bernhard Schink and Dr Aharon Oren for help with the Latin etymology of the new genus name.

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