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ORIGINAL PAPER

Selective isolation and characterisation of novel members of the family *Nocardiopsaceae* and other actinobacteria from a marine sediment of Tioman Island

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Abstract Tioman Island is one of many sources for underexplored actinobacterial diversity in Malaysia. Selective isolation, molecular profiling, 16S rRNA gene sequencing and phylogenetic analyses were carried out to highlight the diversity of the marine actinobacterial community in a sediment collected off Tioman Island. A high number of diverse actinobacteria were recovered using skim milk/HEPES pre-treatment on a mannitol-based medium. A total of 123 actinobacterial strains were isolated, including thirty obligate marine actinobacteria putatively identified as *Salinispora* spp. Molecular fingerprinting profiles obtained with a double digestion approach grouped the remaining non-*Salinispora*-like strains into 24 different clusters, with *Streptomyces* and *Blastococcus* as the major clusters. A total of 17 strains were identified as novel actinobacterial species within the genera *Streptomyces* (n = 6), *Blastococcus* (n = 5), *Marinactinospora* (n = 3), *Nocardiopsis* (n = 1), *Agromyces* (n = 1) and *Nonomuraea* (n = 1) based on 16S rRNA gene sequence analyses. Polyphasic data from three putative *Marinactinospora* spp. showed that the strains represent a new genus in the *Nocardiopsaceae* family. Crude extracts from the strains were also found to inhibit the growth of Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and Gram-negative (*Providencia alcalifaciens*) pathogens. Hierarchical clustering of the bioactivities of an active fraction revealed a unique profile, which is closely related that of fosfomycin.

Keywords *Blastococcus* spp. · Marine actinobacteria · *Marinactinospora* spp.

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Introduction

The *Actinobacteria* lineage justifies considerable attention for their biotechnologically important natural products since the discovery of actinomycin in the 1940s (Waksman and Woodruff 1941). Members of the class *Actinobacteria* are Gram-positive bacteria, most of which contain high G+C content within the genome and are characterised by a homologous insertion of 100 nucleotides between helices 54 and 55 of the 23S rRNA gene (Ventura et al. 2007). Terrestrial originated actinobacteria are extraordinarily diverse, but the ocean that covers three-quarters of the earth's surface has the greatest diversity. The presence of indigenous marine actinobacteria was supported by the discoveries and descriptions of marine species such as *Dietzia maris* (Nesteranko et al. 1982; Rainey et al. 1995), *Rhodococcus marinonascens* (Helmke and Weyland 1984), *Salinibacterium amurskyense* (Han et al. 2003), *Williamsia maris* (Stach et al. 2004) and the first obligate marine genus *Salinispora* (Maldonado et al. 2005). Marine

actinobacteria are mostly derived from marine sediments (54%), followed by sponges (21%), marine invertebrates and sea water (Abdelmohsen et al. 2014).

While culture independent or metagenomic studies are the leading trends in actinobacterial diversity and natural product discoveries, culture dependent studies are essential approaches for cultivation of natural actinobacterial strains that can be screened for biological activities of interests and studied for expression of enzymes and bioactive molecules (Vester et al. 2015). However, it is well established that less than 1% of bacteria can be readily cultivated in vitro (Amann et al. 1995). Fastidious growth requirements, including the need for specific nutrients and growth factors, are among the main obstacles in attempts to cultivate the unculturable (Köpke et al. 2005). Moreover, the dominant species on isolation plates introduce strong interspecies competition for nutrients, which further discourages successful isolation of rare actinobacterial species, causing them to be unculturable. Rare and novel actinobacteria represent unique sources of novel biologically active compounds. Selective isolation techniques using various pre-treatments and incorporation of unusual carbon sources were found to favour the growth of certain taxonomic groups of actinobacteria and encouraged isolation of rare actinobacteria species (Bredholdt et al. 2007; Sun et al. 2010).

Actinobacteria are among saprophytes that exhibit a wide extent of survival and adaptive strategies to persist in natural environments. In certain genera such as *Actinoplanes*, *Dactylosporangium*, *Geodermatophilus*, *Planomonospora* and *Spirillospora*, spores are harboured in sporangia as motile flagellated zoospores (Garrity et al. 1996). Some of the non-spore forming actinobacteria such as *Blastococcus* spp. form motile, single flagellated cells. Production of motile spores and cells enables marine actinobacteria to exhibit chemotaxis and access more nutrient sources. Various selective methods have been employed to isolate zoosporic actinobacteria. These include baiting techniques, chemotactic methods that use organic and/or inorganic nutrients as chemoattractants, and centrifugation methods to improve the total number of actinobacterial isolates originating from terrestrial soil samples (Garrity et al. 1996; Otoguro et al. 2001; Dennis et al. 2013). A flooding solution containing skim milk was postulated to stimulate the motility of spores, thus facilitating the isolation of zoosporic actinobacteria (Suzuki 2001). Actinobacteria are also known to resist high doses of ultraviolet (UV) and high frequency irradiation through production of spores and pigments. Bredholdt et al. (2007) demonstrated the efficiency of ultraviolet irradiation and high frequency irradiation to selectively isolate various actinobacteria from shallow water sediments of the Trondheim fjord, Norway.

Tioman Island, located in the state of Pahang, Malaysia, surrounded by the South China Sea, was reported to be an untapped source of rare marine actinobacteria (Vikineswary et al. 2008). The authors isolated diverse actinobacteria from marine sponges and putatively identified selected isolates as *Actinoplanes* spp., *Micromonospora* spp., *Nocardia* spp., *Polymorphospora* spp., *Pseudonocardia* spp., *Rhodococcus* spp., *Saccharomonospora* spp., *Salinispora* spp., *Sprilliplanes* spp. and *Verrucosispora* spp. Marine actinobacteria were reported to be excellent producers of antimicrobial compounds, in which the backbone of these compounds were synthesized by large enzymes, polyketide synthases and non-ribosomal peptide synthetases (Gomez-Escribano et al. 2016). The objectives of this study were to isolate diverse actinobacteria from a marine sediment sample collected from Tioman Island using various selective cultivation techniques and to investigate the potential antibacterial activity in the isolated strains.

Materials and methods

Sampling and pre-treatment of marine sediment sample

A marine sediment sample was collected on 13 March 2013, from a depth of 7 m by scuba diving, from Pirate Reef, Tioman Island, Pahang, Malaysia (N: 02°49′27.1″, E: 104°09′25.0″). Temperature, salinity and pH of the sampling site were recorded. The sediment sample was homogenized by vigorous vortexing and pre-treated separately using three different methods: (a) UV irradiation (Bredholdt et al. 2007), (b) skim milk/HEPES (0.1% skim milk in 10 mmol/l of HEPES) treatment (modified from Xin et al. 2009) and (c) skim milk/HEPES treatment followed by enrichment in humic acid vitamin broth (HVB) (modified from Xin et al. 2009). Briefly, 200 µl

of skim milk/HEPES treated suspension from (b) was transferred into 1.8 ml HVB and incubated at 28 °C with shaking at 200 rpm for 24 h prior to serial dilution.

Isolation of actinobacteria from marine sediment

Ten-fold serial dilutions and isolation media were prepared using 3% artificial sea water (Instant Ocean®, Aquarium Systems, Sarrebourg, France). The sediment suspension (100 µl) was spread on the surface of three different isolation media: peptone-asparagine agar (M3) (Zhang et al. 2008), mannitol-arginine agar [modified from medium M2 (Zhang et al. 2008)], and humic acid vitamin agar (HVA) (Xin et al. 2009). The mannitol-arginine medium was prepared using 0.5% mannitol, 0.1% L-arginine, 0.1% K_2HPO_4 and 0.05% $MgSO_4$. All isolation media were supplemented with nalidixic acid (15 µg/ml) and nystatin (25 µg/ml) and pH of the media were adjusted to 7.5. All the isolation plates were incubated at 28 °C for up to 8 weeks. Colonies were examined under an inverted microscope. Purified actinobacterial strains were maintained on either modified yeast extract malt extract agar (ISP2, Shirling and Gottlieb 1966) or modified Bennett's agar (MBA) slopes (Tan et al. 2006) and preserved in 20% glycerol suspension at −20 and −80 °C.

Molecular characterisation

Isolated actinobacterial strains were first grouped into *Salinispora*-like and non-*Salinispora*-like strains based on colony colour and presence of aerial mycelium on both ISP2 and MBA plates. Restriction fragment length polymorphism (RFLP) fingerprinting of 16S rRNA gene and its adjacent 16S-23S internal transcribed spacer (ITS) region was used to dereplicate these two groups of marine actinobacterial strains. Four to five days old cultures were used for genomic DNA extraction which was performed with NucleoSpin® Tissue genomic DNA extraction kit (Macherey–Nagel, Germany), according to the manufacturer's instruction. Amplification of the 16S rRNA gene and the adjacent 16S-23S ITS region using the primer pair pA and BL235R (Lanoot et al. 2005) was carried out on all non-*Salinispora*-like strains. Amplicons were digested using *Hae*III (10 U) at 37 °C for 5 min and subsequently with *Bst*U1 (10 U) at 60 °C for 5 min, following the online protocol recommended by New England BioLabs (NEB, Massachusetts, USA; https://nebcloner.neb.com/#!/redigest) and the fragments were resolved on 2% agarose gel electrophoresis in 1 × TAE buffer. The RFLP banding profiles of non-*Salinispora*-like actinobacterial strains were analysed with the BioNumerics software (version 7.1, Applied Maths, Belgium). Bands from 100 to 1000 bp were included for analysis for 16S ITS RFLP fingerprinting using band-based similarity Dice coefficient. Band tolerance positions were set at 0.11%. UPGMA dendogram was derived from the resultant similarity matrixes. The 16S rRNA ITS region of the *Salinispora*-like strains was amplified according to Jensen et al. (1993). The resulted PCR product was subjected to restriction enzyme digestion with 5U of *Ban*I (NEB) and incubated at 37 °C for 15 min, followed by gel electrophoresis on a 2.5% agarose gel (Freel et al. 2012). Amplification of the 16S rRNA gene was carried out as described by Vidgen et al. (2012) using MyTaq™ Red DNA Polymerase (Bioline, UK). Sequencing of the 16S rRNA gene was performed by First Base Laboratories Sdn Bhd (Malaysia) using the BigDye® Terminator v3.1 cycle sequencing kit chemistry. Closest matches were identified using EzBioCloud database (Yoon et al. 2017) and corresponding 16S rRNA gene sequences were retrieved from the database for phylogenetic analysis using MEGA version 6.0 (Tamura et al. 2013). Cut-offs for classification of potential novel taxa were based on 16S rRNA gene sequence similarity values of 98.7% (Sangal et al. 2016). Phylogenetic trees were constructed using neighbour-joining algorithm (Saitou and Nei 1987). Evolutionary distance matrices were generated as described by Tamura (1992) and Tamura and Nei (1993). Bootstrap values were calculated based on 1000 re-samplings (Felsenstein 1985).

Phenotypic characterisation

Colony morphology of the isolated marine actinobacterial strains, including colour of aerial and substrate mycelium and production of diffusible pigmentation, were examined on ISP media number 2 and MBA on day 7 and day 14 of incubation at 28 °C (Shirling and Gottlieb 1966) using the ISCC-NBS colour charts (Kelly 1958). Novel marine actinobacterial strains were tested for their ability to tolerate various growth temperature, pH and sodium chloride. The ISP2

medium served as the basal medium for growth. Growth of purified actinobacterial strains was assessed at ten different temperatures (°C): 4, 10, 15, 20, 25, 32, 37, 45, 50 and 55. The pH range for growth was examined at pH 5.0 to 13.0 in increments of 1 pH unit (Xu et al. 2005). Tolerance to sodium chloride (NaCl) was tested at concentrations up to 10% in increment of 1% NaCl (w/v). Growth of all actinobacterial strains were observed and recorded after 14 days of incubation at 28 °C.

Assessment of antibacterial activity

Antibacterial activity of marine actinobacterial strains were assayed by agar plug diffusion. The strains were cultured on five different production media supplemented with artificial sea salt and 1.5% agar: PM3 (Bredholt et al. 2008), soybean meal glucose (Zheng et al. 2000), MMS (Ismet et al. 2004), Waksman's glucose agar (ATCC medium 241) and SYP (Bose et al. 2015). Agar plates were incubated at 28 °C for up to 21 days. Four test pathogens (*Bacillus subtilis* ATCC 23857, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 47076, *Pseudomonas aeruginosa* ATCC 27853) were cultured at 37 °C for 18 h and the turbidity adjusted to 0.5 McFarland standard prior to use. Lawn cultures of pathogens with the agar plugs were incubated at 37 °C for 18 h. Antibacterial activity was recorded as positive when diameter of inhibition zone was $\geq$ 10 mm. Selected actinobacterial strains which showed potent antibacterial activity against tested pathogens were cultured in soybean meal glucose broth supplemented with 20 g per litre of Amberlite XAD-16 resins. The cultures were incubated at 28 °C for 21 days in an orbital shaker and crude extract was obtained by solvent extraction using dichloromethane: methanol (50:50, v/v). Dried crude extracts were subjected to solid phase extraction (SPE) using step gradient elution with methanol and water mixture from 20:80 to 100:0 and a final column flush with 100% ethyl acetate to yield six fractions. SPE fractions were dried and re-constituted in dimethyl sulfoxide (DMSO) and screened for antibacterial activity against a panel of fifteen pathogens which include Gram-positive pathogens: *B. subtilis* ATCC 23857, *Listeria ivanovii* ATCC BAA-139, *Enterococcus faecium* ATCC 6569, *Staphylococcus epidermidis* ATCC 14990^T, *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA-44 (MRSA) and Gram-negative pathogens: *E. coli* ATCC 47076, *Providencia alcalifaciens* ATCC 9886^T, *Ochrobactrum anthropi* ATCC 49687, *Enterobacter aerogenes* ATCC 35029, *Acinetobacter baumannii* ATCC 19606^T, *P. aeruginosa* ATCC 27853, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ATCC 700720, *Vibrio cholerae* O1 (biotype EI Tor A1552) and *Yersinia pseudotuberculosis* (IP2666 pIBI). Overnight cultures of pathogens were diluted 1:1000 and seeded in a volume of 30 µl/well in sterile clear propylene 384-wells assay plates. Each well was fed with 300 nl of DMSO fractions using a high-throughput pinning robot (Perkin Elmer Janus MDT). Growth curves of pathogens were measured at OD600 at hourly intervals over 24 h in an automated plate reader/shaker (EnVision, Perkin Elmer). DMSO fractions that inhibited Gram-positive and Gram-negative pathogens were selected for parallel screening of twofold dilution series to determine the MIC values. Data were normalized and a BioMap profile was created according to Wong et al. (2012). Normalized MIC values were indicated as 0 or 1. The BioMap profile of the fraction was compared to profiles of training set of antibiotics by hierarchical clustering using Cluster 3.0 software. The cluster plot was displayed as red–black color scheme with a gradient from inactive (black) to most active (red) using TreeView (v1.1.6).

Results

Selective isolation of marine actinobacteria

The marine sediment sample collected from Tioman Island, Pahang, Malaysia had a pH of 7.5 and salinity of 34 parts-per-thousand. Viable colony counts of actinobacteria in the wet sediment sample were in the range of 1.3×10^3 to 3.0×10^4 cfu/g, depending on the type of pre-treatment and isolation media. A total of 123 putative actinobacterial strains was isolated from the marine sediment sample and the strains were initially separated into two groups based on colony morphology. The first group comprised of 76 non-*Salinispora*-like strains, whereas the second group consisted of 47 *Salinispora*-like strains. The non-*Salinispora*-like strains were colonies with black, blue, brown, gray, red, white, yellow, and green coloured aerial mycelia. The *Salinispora*-like strains were bright orange colonies lacking aerial mycelium,

similar to morphological features of the members of the genus *Salinispora*.

A total 114 putative marine actinobacterial strains was isolated using the pre-treatment method with skim milk/HEPES solution (Table 1). This method coupled with the use of a mannitol-based isolation medium and HVA recovered high numbers of actinobacterial strains. This pre-treatment also supported the isolation of high numbers of *Salinispora*-like and non-*Salinispora*-like strains on mannitol-arginine agar (n = 37) and HVA plates (n = 41), respectively (Table 1). The remaining nine strains were recovered from the pre-treatment with UV irradiation (n = 2) and HVB enrichment of skim milk/HEPES treated sample (n = 7) with only four and three strains from mannitol-arginine agar and HVA plates, respectively (data not shown).

Diversity of marine actinobacterial strains

Analyses of the RFLP profiles from 68 non-*Salinispora*-like strains generated 24 clusters (Fig. 1). The 16S rRNA-ITS region amplified by primer pair pA/BL235R has an amplicon size of approximately 1.8 kb and up to ten fragments between 100 bp to 1 kbp were generated from the double RE digestion. The remaining eight non-*Salinispora*-like actinobacterial strains did not produce any amplicons using the primer pair pA/BL235R. Subsequent 16S rRNA gene sequence analyses revealed that the strains were closely related to *Marinactinospora* spp. (n = 3), *Rhodococcus* spp. (n = 2), *Nocardiopsis* spp. (n = 1), *Agromyces* spp. (n = 1) and *Saccharomonospora* spp. (n = 1) (Table 2).

Table 1 Breakdown of the numbers of marine actinobacteria isolated from the Tioman marine sediment sample pre-treated with skim milk/HEPES solution

Isolation medium	Number of non-*Salinispora* strains	Number of *Salinispora*-like strains	Total
Peptone-asparagine agar	15	3	18
Mannitol-arginine agar	14	37	51
Humic acid vitamin agar	41	4	45
Total	70	44	114

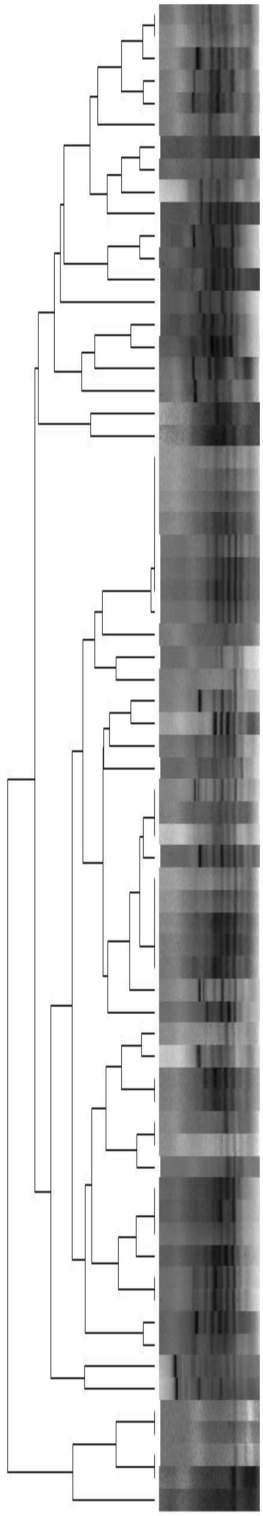
Thirty of the 47 *Salinispora*-like strains profiled using *Ban*I digestion of the 16S-23S ITS amplicons showed the same banding pattern as *Salinispora arenicola* CNH-643^T. The 16S rRNA gene sequences of four randomly selected strains were found to share 100% similarity with the corresponding sequence of *S. arenicola* CNH-643^T. The remaining 17 strains which had different restriction banding profiles from *S. arenicola* CNH-643^T were found to be closely related to *Micromonospora* spp. (n = 8), *Nocardia* spp. (n = 2), *Mycobacterium* spp. (n = 2), *Jishengella* spp. (n = 1), *Plantactinospora* spp. (n = 1), *Pseudonocardia* spp. (n = 1), *Nonomuraea* spp. (n = 1) and *Rhodococcus* spp. (n = 1) based on 16S rRNA gene sequence analyses (data not shown).

Characterisation of novel marine actinobacterial strains

A total of 17 actinobacterial strains were found to represent novel taxa (Table 3, Figs. 2, 3). Fourteen of the novel species were isolated using skim milk/HEPES pre-treated sample, one from HVB enrichment of skim milk/HEPES pre-treated sample and two from sample pre-treated with UV irradiation.

Physiological and morphological characteristics of the novel actinobacterial strains are summarised in Table 3. All strains grew well at pH range from 6 to 12, except for strains TPS3, TPS4, TPS114 and TPS358a which only tolerated up to pH 11. Strains TPS2, TPS3, TPS16, TPS81 and TPS83 were able to grow at pH 5. All strains were able to grow without the presence of NaCl. Only one strain, TPS2, could tolerate up to 10% NaCl (w/v). In terms of temperature, all strains showed good growth at 25, 28, 32 and 37 °C. *Streptomyces* sp. TPS143, TPS137, TPS114 and TPS183 were able to grow at 15 °C or lower. Two strains, TPS183 (*Streptomyces* sp.) and TPS92 (*Agromyces* sp.), were able to grow at 4 °C. In contrast, *Blastococcus* sp. TPS166 and TPS418 as well as strains TPS16, TPS81 and TPS83 were able to grow at 50 °C. One *Blastococcus* strain TPS418 also showed good growth at 55 °C.

Three marine actinobacteria, strains TPS16, TPS81 and TPS83, producing blue aerial mycelia and diffusible pigmentation, were identified to be novel species within the family *Nocardiopseaceae* (Table 3) based on 16S rRNA gene sequence analyses. These strains share 100% similarity of 16S rRNA gene



Strain	Cluster	Closest related species	Similarity
TPS143	1	*Streptomyces specialis* GW41-1564^T	97.96
TPS94	1	*Streptomyces aculeolatus* NBRC 14824^T	100.0
TPS74	1	*Streptomyces mayteni* YIM 60475^T	99.10
TPS122	2	*Streptomyces jiujiangensis* JXJ0074^T	99.29
TPS216	2	*Streptomyces chiangmaiensis* TA4-1^T	98.70
TPS137	3	*Streptomyces sedi* YIM 65188^T	97.56
TPS14	4	*Streptomyces intermedius* NBRC 13049^T	99.72
TPS359	4	*Streptomyces nanshensis* SCSIO01066^T	99.91
TPS38	4	*Streptomyces nanshensis* SCSIO01066^T	99.31
TPS445	4	*Streptomyces nanshensis* SCSIO01066^T	99.61
TPS24	5	*Streptomyces jiujiangensis* JXJ0074^T	99.13
TPS41	5		
TPS63	5	*Streptomyces violascens* ISP 5183^T	100.0
TPS35	6	*Streptosporangium amethystogenes* subsp. *fukuiense* JCM 10083^T	99.39
TPS27	7	*Streptomyces hydrogenans* NBRC 13475^T	99.91
TPS42	7		
TPS181	7	*Streptomyces samsunensis* M1463^T	99.72
TPS180	7	*Streptomyces carpaticus* NBRC 15390^T	99.24
TPS37	8	*Saccharopolyspora hirsuta subsp. hirsuta* ATCC 27875^T	98.87
TPS89	8	*Actinomadura livida* JCM 3387^T	100.0
TPS198	9	*Streptomyces hydrogenans* NBRC 13475^T	99.91
TPS199	9		
TPS201	9		
TPS202	9		
TPS65	9		
TPS67	9		
TPS68	9		
TPS66	9		
TPS208	10	*Streptomyces thermocarboxydus* DSM 44293^T	99.31
TPS3	11	*Streptomyces caeruleatus* NRRL B-24802^T	97.30
TPS5	11		
TPS11	12	*Streptomyces xiamenensis* MCCC 1A01550^T	99.44
TPS364	12	*Streptomyces xiamenensis* MCCC 1A01550^T	99.36
TPS6	12	*Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T	98.92
TPS15	12	*Streptomyces harbinensis* NEAU-Da3^T	99.47
TPS1	13	*Streptomyces collinus* NBRC 12759^T	99.01
TPS48	13		
TPS60	13	*Streptomyces somaliensis* DSM 40738^T	100.0
TPS183	13	*Streptomyces harbinensis* NEAU-Da3^T	98.85
TPS43	14		
TPS44	14		
TPS45	14		
TPS46	14		
TPS47	14		
TPS4	15	*Streptomyces carpaticus* NBRC 15390^T	98.12
TPS53	16	*Streptomyces wuyuanensis* CGMCC 4.7042^T	99.10
TPS10	17	*Streptomyces spongiicola* HNM0071^T	99.51
TPS75	17	*Streptomyces harbinensis* NEAU-Da3^T	99.65
TPS210	18	*Streptomyces albidoflavus* DSM 40455^T	99.82
TPS211	18		
TPS51	19	*Streptomyces spongiicola* HNM0071^T	99.21
TPS8	19		
TPS209	19	*Streptomyces spongiicola* HNM0071^T	99.30
TPS12	20	*Streptomyces daghestanicus* NRRL B-5418^T	99.92
TPS17	20	*Streptomyces jiujangensis* JXJ0074^T	99.56
TPS7	20		
TPS31	20	*Streptomyces daghestanicus* NRRL B-5418^T	99.49
TPS58	21	*Streptomyces daghestanicus* NRRL B-5418^T	100.0
TPS61	21		
TPS114	22	*Streptomyces karpasiensis* K413^T	97.79
TPS358a	22	*Nonomuraea salmonea* DSM 43678^T	98.09
TPS419	23		
TPS77	23	*Streptomyces cheonanensis* VC-A46^T	99.47
TPS357	24	*Blastococcus saxobsidens* BC448^T	97.94
TPS418	24	*Blastococcus saxobsidens* BC448^T	97.97
TPS166	24	*Blastococcus endophyticus* YIM 68236^T	98.31
TPS448	24	*Blastococcus endophyticus* YIM 68236^T	96.19
TPS459	24	*Blastococcus saxobsidens* BC448^T	98.03

◀**Fig. 1** Banding profiles based on RFLP of 16S–23S ITS region of non-*Salinispora*-like strains. Representative strains from each cluster were selected for 16S rRNA gene sequencing. The closest related species match and the percentage of similarity (%) are listed

sequence (1399–1433 bp) between each other and below 98.7% similarities to the closest species, *Marinactinospora thermotolerans* SCSIO00652^T. All three strains formed a distinct and tight cluster within members of the *Nocardiopsaceae* family in the neighbour-joining phylogenetic tree (Fig. 3).

Assessment of antibacterial activity

A total of 13 strains from the *Streptomyces* cluster excluding the novel species were observed to exhibit antibacterial activity against at least one pathogen (Table 4). Strains TPS6, TPS10, TPS12 and TPS17 inhibited the growth of Gram-negative as well as Gram-positive pathogens. Strains TPS10, TPS12 and TPS17 growing on Waksman's glucose agar and SYP media inhibited the growth of *E. coli* ATCC 47076 and *S. aureus* ATCC 29213. Another *Streptomyces* strain TPS6 inhibited the growth of *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857. Strain TPS37 which was closely related to *Saccharopolyspora hirsuta* subsp. *hirsuta* ATCC 27875^T was able to inhibit *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857 when cultured on PM3.

The orange coloured strains TPS111 and TPS121 were among the *Salinispora*-like species that exhibited antibacterial activity. Both strains were identified to be *Micromonospora* spp. that inhibited the growth of Gram-positive *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857. Strain TPS111 produced activity on four of the five production media, whereas strain TPS121 produced activity only on SYP. All the 30 strains which were highly similar to *S. arenicola* CNH-643^T exhibited antibacterial activity against Gram-positives *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857. A total of 24 strains produced antibacterial activity on all five production media. Interestingly, strain TPS178 showed positive inhibitory activity against Gram negative *P. aeruginosa* ATCC 27853. Another strain TPS104 also inhibited the growth of *P. aeruginosa* ATCC 27853 along with the Gram-positive *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213 when it was cultured on Waksman's glucose medium and SYP; however, it inhibited only *S. aureus* ATCC 29213 when cultured on PM3 and soybean meal glucose medium (Table 4).

Among the 17 novel strains, the *Streptomyces* spp. TPS114 and TPS137 were active against *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213, whereas strain TPS143 was only active against *B. subtilis* ATCC 23857 (Table 4). All three strains produced antibacterial activity on production medium MMS. Three strains belonging to the family *Nocardiopsaceae*, TPS16, TPS81, and TPS83, grown on soybean meal glucose medium, were shown to exhibit antibacterial activity against *E. coli* ATCC 47076, *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213. The strain TPS83 was thus selected for further study based on novelty and spectrum of activities. Examination of the crude SPE fractions of TPS83 revealed the antibacterial potential of this novel strain. The fraction TPS83_D eluted with 80% methanol–water appeared to be the most potent among the six fractions as it inhibited the growth of Gram-positive pathogens and

Table 2 Sequence match for non-*Salinispor*a-like actinobacterial strains which did not produce 16S rRNA-ITS amplicons

Strain	Closest related species	Family	Similarity (%)
TPS2	*Nocardiopsis alba* DSM 43377^T	*Nocardiopsaceae*	98.22
TPS16	*Marinactinospora thermotolerans* SCSIO00652^T	*Nocardiopsaceae*	97.11
TPS81	*Marinactinospora thermotolerans* SCSIO00652^T	*Nocardiopsaceae*	96.75
TPS83	*Marinactinospora thermotolerans* SCSIO00652^T	*Nocardiopsaceae*	96.91
TPS33	*Rhodococcus equi* NBRC 101255^T	*Nocardiaceae*	100.0
TPS179	*Rhodococcus equi* NBRC 101255^T	*Nocardiaceae*	99.78
TPS92	*Agromyces aurantiacus* YIM 21741^T	*Microbacteriaceae*	98.29
TPS125	*Saccharomonospora xinjiangensis* XJ-54^T	*Pseudonocardiaceae*	99.70

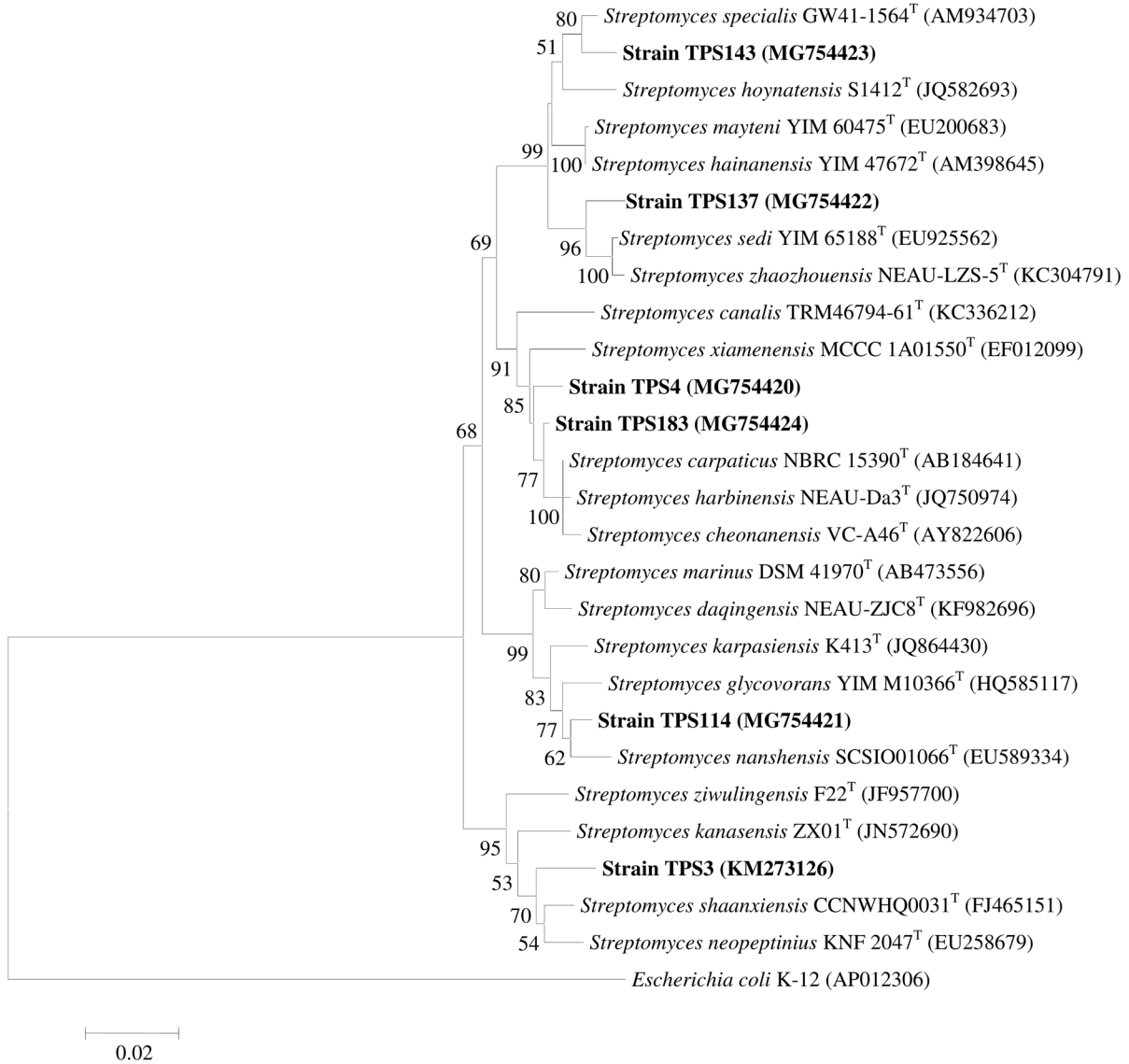


Fig. 2 Neighbour-joining tree based on almost full length 16S rRNA gene sequences of strains TPS3, TPS4, TPS114, TPS137, TPS143, TPS183 and closely related *Streptomyces* spp. Evolutionary distances were computed using Tamura 3-parameter method (Tamura 1992). Bootstrap values are denoted at nodes on branches based on 1000 re-sampling. Only values higher or equal to 50% are indicated. Bar represents 2% sequence divergence

Gram-negative *P. alcalifaciens* ATCC 9886^T. MIC values of crude SPE fraction TPS83_D were determined as 100 µM for *B. subtilis* ATCC 23857, *E. faecium* ATCC 6569, *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA-44, 25 µM for *S. epidermidis* ATCC 14990^T and 6.25 µM for *P. alcalifaciens* ATCC 14990^T by parallel screening of the twofold dilution series of the fraction. The BioMap profile of fraction TPS83_D formed a distinct cluster from the training set of antibiotics suggesting a different antibiotic profile (Fig. 4).

Discussion

Selective isolation of diverse actinobacteria

In this study, skim milk/HEPES pre-treatment with centrifugation at 1000×*g* was shown to improve

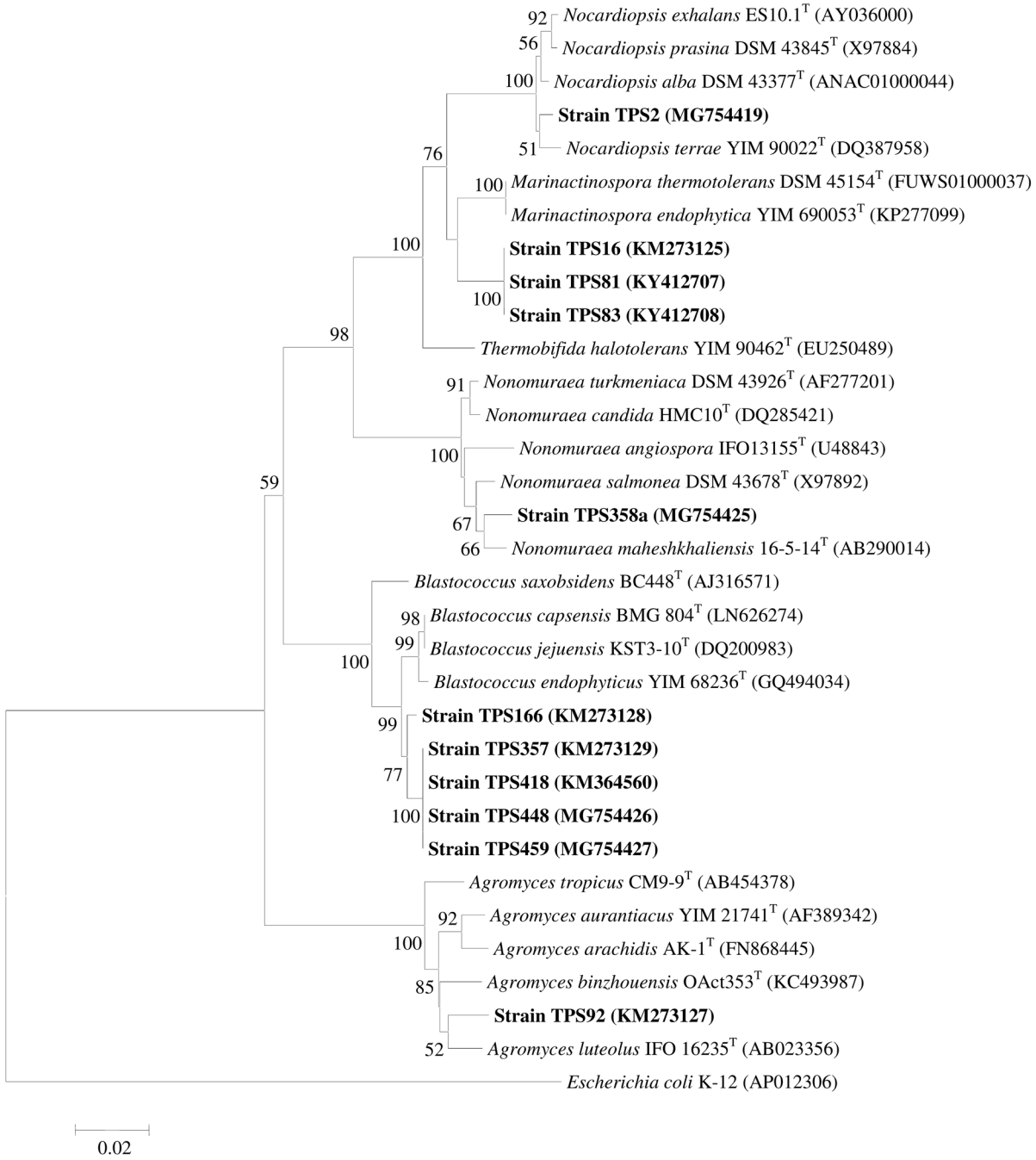


Fig. 3 Neighbour-joining tree based on almost full length 16S rRNA gene sequences of eleven novel strains and closely related members of the families *Geodermatophilaceae*, *Microbacteriaceae, Nocardiopsaceae* and *Streptosporangiaceae*. Evolutionary distances were calculated using the algorithm of Tamura-Nei model. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling. Only values higher or equal to 50% are indicated. Bar represents 2% sequence divergence

isolation of diverse actinobacteria from the sediment sample, yielding members of 18 actinobacterial genera. Shannon–Wiener index (H') for the pre-treated sample indicated that mannitol-arginine agar and HVA recovered greater diversity of actinobacteria than peptone-asparagine agar (Fig. 5). By coupling

Table 3 Novel actinobacterial strains isolated from a Tioman marine sediment sample

Strain	Fingerprinting cluster	Colour of aerial mycelia	Diffusible pigment	Temp (°C)	pH	NaCl (%)	Closest related species	Similarity (%)	Accession number
TPS143	1	Light brownish gray	Light brown	15–37	6–12	0–4	*Streptomyces specialis* GW41-1564^T	98.07	MG754423
TPS137	3	Light yellow	Pale pink	15–37	6–12	0–7	*Streptomyces sedi* YIM 65188^T	97.65	MG754422
TPS3	11	Pale yellow	Moderate yellow	20–37	5–11	0–6	*Streptomyces ziwulingensis* F22^T	96.76	KM273126
TPS183	13	Medium gray	Greyish yellowish brown	4–45	6–12	0–8	*Streptomyces harbinensis* NEAU-Da3^T	97.47	MG754424
TPS4	15	Light brownish gray	Absent	20–32	6–11	0–4	*Streptomyces carpaticus* NBRC 15390^T	97.80	MG754420
TPS114	22	Moderate yellow	Absent	15–32	6–11	0–6	*Streptomyces karpasiensis* K413^T	98.34	MG754421
TPS358a	22	Strong purplish red	Absent	20–37	6–11	0–3	*Nonomuraea turkmeniaca* DSM 43926^T	97.57	MG754425
TPS166	24	Moderate red	Moderate yellow	15–50	6–12	0–8	*Blastococcus endophyticus* YIM 68236^T	97.65	KM273128
TPS357	24	Vivid yellowish pink	Absent	10–45	6–12	0–8	*Blastococcus endophyticus* YIM 68236^T	97.84	KM273129
TPS448	24	Deep yellowish pink	Absent	10–45	6–12	0–7	*Blastococcus saxobsidens* BC448^T	98.39	MG754426
TPS459	24	Deep yellowish pink	Absent	10–45	6–12	0–7	*Blastococcus saxobsidens* BC448^T	98.39	MG754427
TPS418	24	Deep yellowish pink	Absent	10–55	6–12	0–8	*Blastococcus jejuensis* KST3-10^T	97.64	KM364560
TPS2	Not classified	Strong yellow	Absent	15–32	5–12	0–10	*Nocardiopsis alba* DSM 43377^T	97.08	MG754419
TPS16	Not classified	Strong blue	Moderate blue	20–50	5–12	0–8	*Marinactinospora thermotolerans* SCSIO 00652^T	96.94	KM273125
TPS81	Not classified	Strong blue	Moderate blue	20–50	5–12	0–8	*Marinactinospora thermotolerans* SCSIO 00652^T	97.07	KY412707
TPS83	Not classified	Strong blue	Moderate blue	20–50	5–12	0–8	*Marinactinospora thermotolerans* SCSIO 00652^T	96.63	KY412708
TPS92	Not classified	Pale yellow	Absent	4–45	6–12	0–8	*Agromyces humatus* CD5^T	97.20	KM273127

Almost full length 16S rRNA gene sequences (> 1400 bp) were used to align with corresponding sequences of closely related species using CLUSTAL_W. BLAST searches were performed on the EzBioCloud server. Physiological and morphological characteristics were observed on yeast extract-malt extract agar (ISP2) plates as basal medium

Table 4 Antibacterial activity of selected actinobacterial strains. Strains were cultured on five different production media and the activity was assessed by agar plug diffusion assay

Strain	Family/genus	1	2	3	4	5
Non-*Salinispora* like isolates: novel actinobacterial isolates						
TPS16	*Nocardiopsaceae*	Bs Sa	Bs Sa Ec	Bs Sa	–	–
TPS81	*Nocardiopsaceae*	–	Bs Sa Ec	Bs Sa Ec	Bs Sa	Bs Sa
TPS83	*Nocardiopsaceae*	Bs Sa	Bs Sa Ec	Bs	–	–
TPS114	*Streptomyces* sp.	Bs	Bs	Bs Sa	Bs Sa	Bs Sa
TPS137	*Streptomyces* sp.	–	–	Sa	Bs	–
TPS143	*Streptomyces* sp.	Bs	–	Bs	–	–
Non-*Salinispora* like isolates: the *Streptomyces* cluster						
TPS1	*Streptomyces* sp.	–	–	Bs	Bs Sa	Bs
TPS6	*Streptomyces* sp.	–	–	Bs Sa Pa	–	Sa
TPS10	*Streptomyces* sp.	–	Bs Sa	–	Ec Sa	Ec
TPS12, TPS17	*Streptomyces* sp.	–	–	–	Ec Sa	Ec Sa
TPS14	*Streptomyces* sp.	Bs Sa	–	–	–	–
TPS38	*Streptomyces* sp.	–	–	–	Sa	–
TPS51	*Streptomyces* sp.	–	–	–	Bs Sa	–
TPS94	*Streptomyces* sp.	–	Bs Sa	Bs Sa	Bs Sa	Bs Sa
TPS181	*Streptomyces* sp.	–	Sa	–	Sa	–
TPS216	*Streptomyces* sp.	–	–	–	Bs Sa	–
TPS445	*Streptomyces* sp.	–	Bs	Bs Sa	–	–
TPS37	*Saccharopolyspora* sp.	Bs Sa	–	–	–	–
Salinispora-like isolates: others						
TPS111	*Micromonospora* sp.	Bs Sa	Bs Sa	Bs Sa	–	Bs Sa
TPS121	*Micromonospora* sp.	–	–	–	–	Bs
Salinispora-like strains: the *Salinispora* cluster						
TPS101, TPS103, TPS107	*Salinispora* sp.	Sa	Sa	Bs Sa	Sa	Sa
TPS102	*Salinispora* sp.	Sa	Sa	Sa	Bs Sa	Sa
TPS104	*Salinispora* sp.	Sa	Sa	Bs Sa	Bs Sa Pa	Bs Sa Pa
TPS105, TPS108, TPS109, TPS112, TPS123, TPS126, TPS127, TPS146, TPS147, TPS148, TPS158	*Salinispora* sp.	Sa	Sa	Sa	Sa	Sa
TPS113, TPS132, TPS167, TPS355	*Salinispora* sp.	Sa	–	Sa	Sa	Sa
TPS115	*Salinispora* sp.	Bs Sa	Sa	Sa	Bs Sa	Bs Sa
TPS118	*Salinispora* sp.	–	–	Bs Sa	Bs Sa	Bs Sa
TPS119	*Salinispora* sp.	–	Sa	Bs Sa	–	Bs Sa
TPS120	*Salinispora* sp.	Sa	–	Bs Sa	Bs Sa	Sa
TPS135	*Salinispora* sp.	Bs Sa	Sa	–	Bs Sa	Bs Sa
TPS142	*Salinispora* sp.	Sa	–	–	Sa	Sa
TPS153	*Salinispora* sp.	Bs Sa	Sa	Sa	Sa	Bs Sa
TPS174	*Salinispora* sp.	Bs Sa	Sa	Bs Sa	Sa	Bs Sa
TPS178	*Salinispora* sp.	Sa	–	Sa Pa	Sa	Sa
TPS335	*Salinispora* sp.	Sa	Sa	Sa	Sa	Bs Sa

Bs, *B. subtilis* ATCC 23857; Sa, *S. aureus* ATCC 29213; Ec, *E. coli* ATCC 47076; Pa, *Pseudomonas aeruginosa* ATCC 27853; *1* PM3, *2* Soybean meal glucose, *3* MMS, *4* Waksman's glucose agar, *5* SYP. Antibacterial activity was considered positive when the diameter of inhibition zone was at least 10 mm. Absence of antibacterial activity and diameter of inhibition zones smaller than 10 mm are indicated by "–"

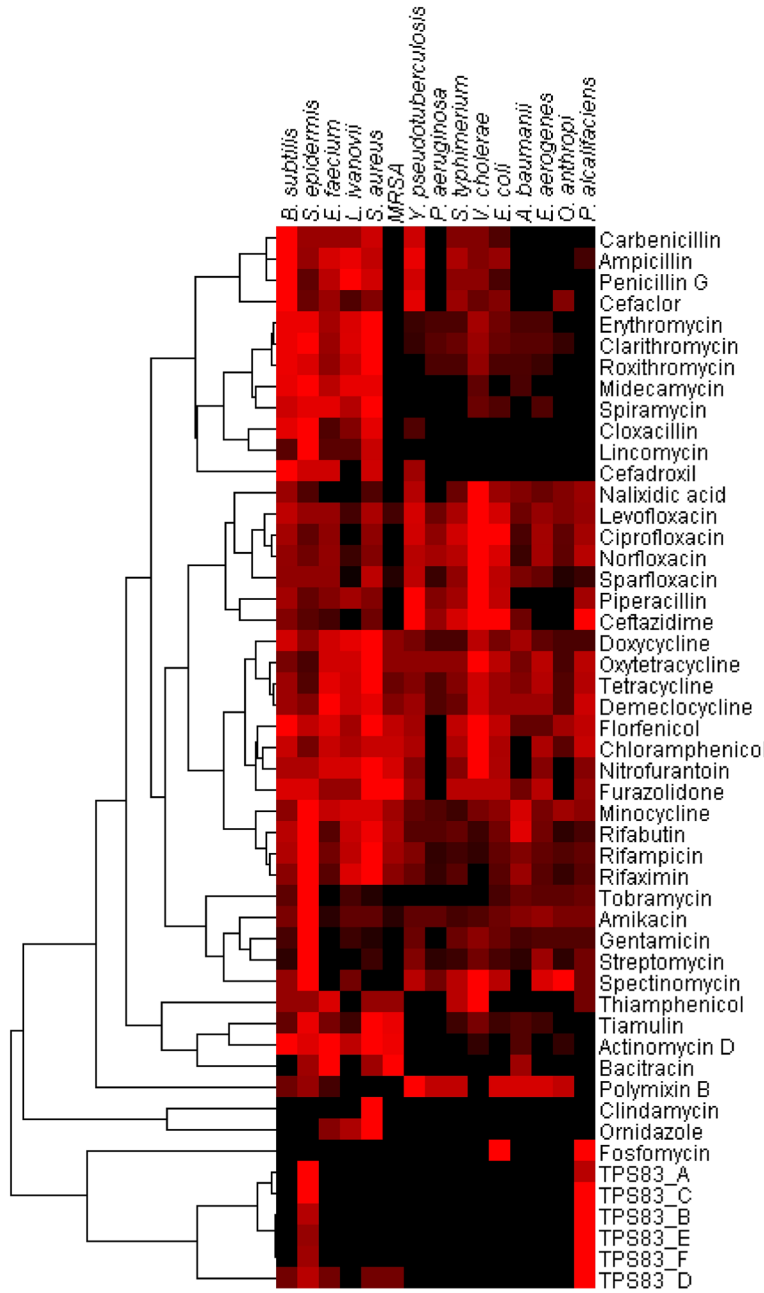


Fig. 4 Fraction TPS83_D was found to be the most active among the six fractions, showing inhibitory activity against six out of 15 tested pathogens. Hierarchical clustering of active fractions with reference antibiotics based on normalized MIC values revealed a single and distinct cluster of all TPS83 crude fractions, which was closely related to fosfomycin based on the activity profile. Potency of fractions was represented by a red–black colour scheme: inactive (black) and most active (red). (Color figure online)

skim milk treatment with centrifugation at 1000–1500 × *g*, numbers of non-motile actinobacteria and *Streptomyces* spp. colonies decreased, thereby facilitating isolation of rare actinobacteria (Suzuki et al. 1999; Hayakawa 2008). However, the HVB enrichment of skim milk/HEPES treated sample encouraged the growth of higher populations of fast growing non-actinobacterial strains. Thus, the recovery rate of actinobacteria was much lower compared to the skim milk/HEPES pre-treatment without enrichment in HVB. Humic acid vitamin agar was found to support growth of non-*Salinispora*-like strains. Humic acid is a sole carbon and nitrogen source that encourages growth of spore-forming actinobacteria including various rare actinobacteria,

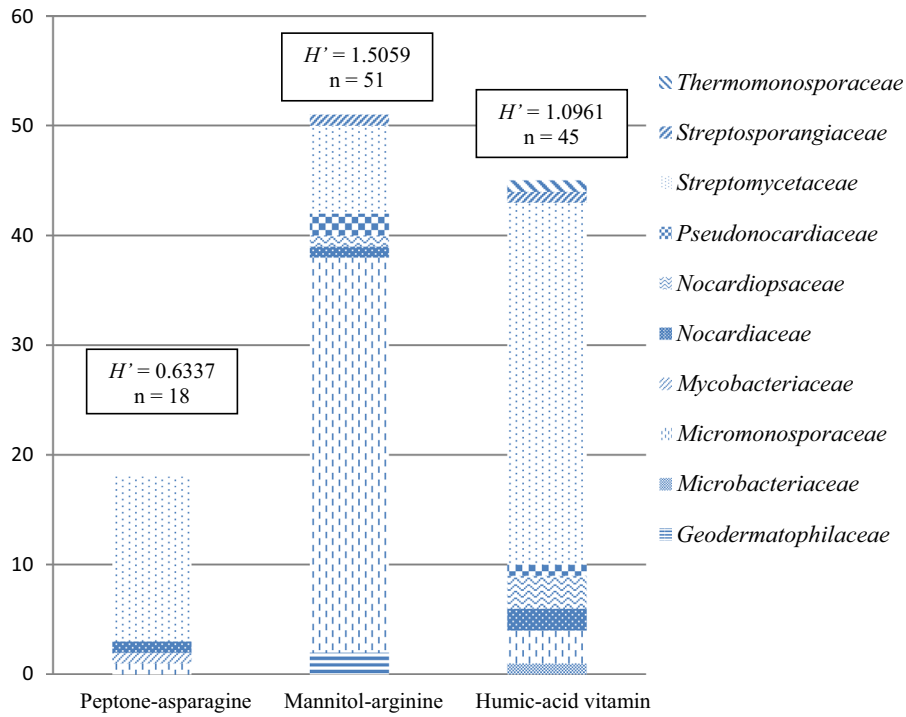


Fig. 5 Diversity of actinobacteria recovered from Tioman marine sediment following skim milk/HEPES treatment on peptone-asparagine, mannitol-arginine and humic acid vitamin agars. Shannon–Wiener index (H') and total number of actinobacterial strain (n) isolated from each isolation medium are also indicated

while reducing growth of non-filamentous bacterial colonies (Hayakawa 2008).

In addition, most of the *Salinispora*-like strains were isolated using a mannitol-based medium modified for this study. Isolation medium with low concentrations of mannitol had been reported to yield marine strains including *Salinispora* spp. from marine sediments (Jensen et al. 2005). Highly UV-resistant actinobacteria of the genera *Arthrobacter*, *Curtobacterium* and *Geodermatophilus* were isolated from desert rock samples treated with UV irradiation (Kuhlman et al. 2005). These actinobacteria were found to appear in clusters or aggregates. In this study, the only strains recovered from the UV irradiation pre-treatment method were *Blastococcus* spp. which were observed as irregularly shaped coccoid cell aggregates.

Diversity and characterisation of isolated actinobacteria

In general, analyses of the 16S rRNA gene sequences of marine actinobacterial strains indicated close relationships to members of 18 genera: *Actinomadura*, *Agromyces*, *Blastococcus*, *Jishengella*, *Marinactinospora*, *Micromonospora*, *Mycobacterium, Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Plantactinospora*, *Pseudonocardia,* *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinispora*, *Streptomyces*, and *Streptosporangium*. Almost half and a quarter of the total actinobacteria isolated were *Streptomyces* spp. (47.97%) and *Salinispora* spp. (23.58%), respectively.

The non-*Salinispora*-like strains were grouped into two major clusters: the *Streptomyces* cluster (Clusters 1–5, Cluster 7, Clusters 9–21, Cluster 23) and the *Blastococcus* cluster (Cluster 24) (Fig. 1). The *Streptomyces* cluster was found to share less than 10 RFLP banding patterns suggesting high diversity between the isolated strains of this genus. Analyses of 16S rRNA gene sequences indicated that *Streptomyces* spp. were isolated in a high number on mannitol-arginine and HVA media. Strains of *Salinispora* spp. and *Blastococcus* spp. were recovered exclusively from mannitol-arginine agar regardless of pre-treatments. Genera that were also recovered from mannitol-arginine agar included *Nonomuraea*, *Saccharomonospora*, *Nocardiopsis*, *Plantactinospora* and *Pseudonocardia*. Although the *Plantactinospora* spp. isolated from this study shared 99.4% similarity

of 16S rRNA gene sequence to *Plantactinospora endophytica* YIM 68255^T, this is the first report on isolation of *Plantactinospora* sp. from a marine sediment.

All five strains in the *Blastococcus* cluster (strains TPS166, TPS357, TPS418, TPS448 and TPS459) were putatively identified as novel species based on 16S rRNA sequence analyses. There are only five *Blastococcus* species validly named to date: *Blastococcus aggregatus*, *Blastococcus capsensis*, *Blastococcus endophyticus*, *Blastococcus jejuensis* and *Blastococcus saxobsidens*, which were isolated from brackish water (Ahrens and Moll 1970), archaeological Roman pool (Hezbri et al. 2016), medicinal plant leaves (Zhu et al. 2013), beach sediment (Lee 2006) and monument stones (Urzì et al. 2004), respectively. This study demonstrated the first isolation of members of the genus *Blastococcus* from a marine sediment sample. The strains were able to tolerate up to 8% NaCl; strains TPS166 and TPS418 showed growth up to 50 and 55 °C, respectively. In contrast, known *Blastococcus* species are only capable of tolerating up to 3% NaCl and 45 °C. Moreover, all five novel marine *Blastococcus* spp. derived from the Tioman marine sediment sample were able to grow from pH 6 to 12, whereas *B. saxobsidens* and *B. jejuensis* were reported to only tolerate up to pH 8 and pH 10, respectively.

Members within the family of *Nocardiopsaceae* are known to be halophiles or halotolerant species that tolerated 10% NaCl or above, as showed by members of the genera *Haloactinospora* and *Salinactinospora* (Tang et al. 2008; Chang et al. 2012). The type genus *Nocardiopsis* also contains alkaliphilic members such as *Nocardiopsis valliformis* and *Nocardiopsis dassonvillei* subsp. *prasina* that tolerate up to pH 13 (Miyashita et al. 1984; Yang et al. 2008). Members of the family *Nocardiopsaceae* are commonly present in terrestrial soil, however, the genus *Spinactinospora* was only discovered from marine sediments and the type species *Spinactinospora alkalitolerans* is known to be alkaliphilic (Chang et al. 2012). In this study, four new strains belonging to the *Nocardiopsaceae* family were isolated from the Tioman marine sediment: strain TPS2 is closely related to *Nocardiopsis alba* and strains TPS16, TPS81 and TPS83 were closely related to *M. thermotolerans*. These strains were characterised by their ability to tolerate up to pH 12 and 8-10% NaCl.

Overall, 17 novel species of actinobacteria were isolated from the sediment sample, constituting 21.5% of the total number of non-*Salinispora*-like strains and 13.5% of the total actinobacterial strains, indicating that marine sediments of Tioman Island is indeed a potential resource of novel and diverse actinobacteria.

Antibacterial activity of novel members of the family *Nocardiopsaceae*

Novel actinobacteria of the *Nocardiopsaceae* family, represented by the strains TPS16, TPS81 and TPS83, were recovered from HVA following skim milk/HEPES pre-treatment of the marine sediment sample. The novel strains cultured on soybean meal glucose medium were shown to be able to inhibit the growth of Gram-negative *E. coli* ATCC 47076 as well as Gram-positives *B. subtilis* and *S. aureus* ATCC 29213. Although all three strains shared 100% similarity of their 16S rRNA gene sequences, significantly different antibacterial activity profiles were obtained when cultured different production media. Strains TPS16 and TPS83 were able to inhibit both *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213 while strain TPS81 did not show any antibacterial activity when grown on PM3. In contrast, strain TPS81 was able to inhibit the same pathogens when grown on Waksman's glucose agar and SYP but strains TPS16 and TPS83 could not. These results represent the possibility of producing strain-specific antibacterial compounds.

Hierarchical clustering of the active fractions obtained from the crude extract of strain TPS83 with BioMap profiles revealed that all the fractions formed a separate and distinct cluster although closely related to fosfomycin. The antibiotic fosfomycin is only one of the few antibiotics that still remained active against broad spectrum targets including the multi-drug resistant and extensively-drug resistant pathogens (Falagas et al. 2016). It is a bactericidal compound that interferes with the formation of UDP *N*-acetylmuramic acid, the peptidoglycan precursor, which is involved in the first cytoplasmic step of bacterial cell wall synthesis (Borisova et al. 2014). Fraction TPS83_D was shown to inhibit the largest number of pathogens, including Gram-negative *P. alcalifaciens* ATCC 14990^T, as compared to other fractions. These results suggest potential novel antibacterial activity of the fractions and further studies are needed to isolate the bioactive compounds.

Conclusion

Culture dependent techniques are important in recovering bioactive actinobacteria from marine environmental samples. Our study demonstrated successful selective isolation of high numbers and diverse marine actinobacteria from a marine sediment including 17 novel actinobacterial strains based on skim milk/HEPES pre-treatment using mannitol-based and humic acid vitamin media. Future studies are ongoing to describe the novel species and to identify their potentially novel antibacterial metabolites.

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