Streptomyces kebangsaanensis sp. nov., an endophytic actinomycete isolated from an ethnomedicinal plant, which produces phenazine-1-carboxylic acid

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A spore-forming streptomycete designated strain SUK12T was isolated from a Malaysian ethnomedicinal plant. Its taxonomic position, established using a polyphasic approach, indicates that it is a novel species of the genus Streptomyces. Morphological and chemical characteristics of the strain were consistent with those of members of the genus Streptomyces. Analysis of the almost complete 16S rRNA gene sequence placed strain SUK12T in the genus Streptomyces where it formed a distinct phyletic line with recognized species of this genus. The strain exhibited highest sequence similarity to Streptomyces corchorusii DSM 40340T (98.2%) followed by Streptomyces chrestomyceticus NRRL B-3310T (98.1%). The G+C content of the genomic DNA was 74 mol%. Chemotaxonomic data [MK-9(H8) as the major menaquinone; LL-diaminopimelic acid as a component of cell-wall peptidoglycan; C12:0, C14:0, C15:0 and C17:1 as the major fatty acids; phospholipid type II] supported the affiliation of strain SUK12T to the genus Streptomyces. The results of the phylogenetic analysis and phenotypic data derived from this and previous studies allowed the genotypic and phenotypic differentiation of strain SUK12T from the related species of the genus Streptomyces. The DNA–DNA relatedness value between strain SUK12T and S. corchorusii DSM 40340T is 18.85 ± 4.55%. Strain SUK12T produces phenazine-1-carboxylic acid, known as tubermycin B, an antibacterial agent. It is proposed, therefore, that strain SUK12T (=DSM 42048T=NRRL B-24860T) be classified in the genus Streptomyces as the type strain of Streptomyces kebangsaanensis sp. nov.

The genus Streptomyces is the largest genus in the family Streptomycetaceae of the phylum Actinobacteria (Kämpfer, 2006). In 2012, 605 described species were encompassed by the genus Streptomyces (Euzéby, 2012). The identification of described species is facilitated by the use of 16S rRNA gene sequencing information when compared against the rapid expansion of sequences deposited online (Macrae, 2000).
During our routine screening for endophytic antibiotic-producing micro-organisms from ethnomedicinal plants from several parts of Malaysia, an actinomycete that produced the antibiotic phenazine-1-carboxylic acid, known as tubercidin, was isolated. This strain, designated SUK12T, demonstrated a colonial morphology consistent with its assignment to the genus Streptomyces. The aim of the present investigation was to determine the taxonomic position of this organism, based on a full polyphasic taxonomic study.

Strain SUK12T was isolated from the internal tissue of the stem of an ethnomedicinal plant, Portulaca oleracea L. (local name, Gelang pasir). The plant was collected from sandy land at the Nenasi Reserve Forest, Pahang Malaysia. The plant sample was subjected to surface sterilization procedures to eliminate epiphytes by dipping the plant sequentially in 95% (v/v) ethanol, 3.125% (v/v) sodium hypochlorite and 95% (v/v) ethanol, for 1 min, 6 min and 30 s, respectively, followed by rinsing with sterile water (Coombs & Franco, 2003). The outer layer of the plant stem was removed and the inner tissue was excised and plated onto several isolation media. The pH of all media was adjusted to 7.2 and the media were supplemented with nystatin (50 μg ml⁻¹) and cycloheximide (50 μg ml⁻¹) to inhibit the growth of fungi. The plates were kept at 27 °C for the isolation of the endophyte. After 2 weeks of incubation, a streptomycete-like strain, labelled SUK12T, emerged from the Actinomycete Isolation Agar medium (Dişco). This organism was isolated, purified and maintained on yeast extract–malt extract agar [International Streptomyces Project (ISP) medium 2; Shirling & Gottlieb, 1966] at 4 °C. The strain was stored in 20% (v/v) glycerol at −80 °C.

Morphological characteristics of strain SUK12T were observed as described by Shirling & Gottlieb (1966) on eight media, namely ISP 2, ISP 3, ISP 4, ISP 5, ISP 7, Bennett’s agar, potato dextrose agar (PDA) and nutrient agar (Atlas, 1993). The spore-chain morphology of strain SUK12T was examined by light microscopy (CX31RBSF, Olympus) and scanning electron microscopy (SEM; SUPRA 55VP, Zeiss) of 14-day-old cultures grown on ISP 2. Aerial spore-mass colour, substrate mycelia pigmentation and the production of diffusible pigments were recorded on a number of agar media incubated at 28 °C for 14 days. The colour was recorded by comparing the cultures with the most suitable colour chips from the ISCC-NBS colour charts (Kelly, 1964).

Degradation of casein, starch, tyrosine and xanthine and lysozyme resistance were determined according to the protocols of Gordon et al. (1974). Growth in the presence of sodium chloride was determined according to the method of Tresner et al. (1968) and growth on 10 sole carbon sources was determined on carbon utilization agar (ISP 9) as described by Shirling & Gottlieb (1966). Strain SUK12T was also examined for its ability to grow in the presence of 12 antibiotics; 6 mm sensitivity discs (Dişco) were placed centrally on plates of ISP 2 seeded with a loopful of a 14-day-old ISP 2 broth culture and inhibition zones observed after 2–5 days of incubation at 28 °C. Temperature and pH requirements were determined on ISP 2 agar plates and antimicrobial activity of strain SUK12T was examined using a streak plate technique. Strain SUK12T and the most closely related type strain Streptomyces corchorusii DSM 40340T were incubated at 10, 28, 30, 37 and 45 °C for 14 days. The pH of the agar medium was adjusted after autoclaving using a sterile pH meter to pH 3.5 to 12.0 at 0.5-unit intervals. Strain SUK12T was streaked straight across the dish of ISP 2 agar plates and incubated at 28 °C for 14 days. The test pathogenic bacteria and fungi were inoculated perpendicular to the antagonist and incubated for 24 h at 37 °C for bacteria. Filamentous fungi were incubated at 28 °C for 96 h. Growth inhibition was determined by measuring the inhibition zone from the antagonist.

Biomass was prepared by culturing strain SUK12T at 30 °C in modified Sauton’s broth medium (Mordarska et al., 1972) for 10 days. The culture was centrifuged at 12 000 g and the pelleted cells were washed two times with sterile distilled water and dried using a freeze dryer (Minnikin et al., 1984). The isomeric form of diaminopimelic acid (Apm) of strain SUK12T was determined according to the method of Stanek & Roberts (1974). Menaquinones and phospholipids were extracted from freeze-dried biomass (about 100 mg) and examined using the integrated method of Minnikin et al. (1984). Menaquinones were analysed by direct injection into a reverse-phase LC-MS mirOTOF-Q (Bruker) where atmospheric-pressure chemical ionization-positive and -negative modes were employed. The probe voltage was 4.0 kV, probe temperature was 200 °C and the scan range from 50 to 1000 m/z. For the analysis of whole-cell fatty acids, cells of strain SUK12T and S. corchorusii DSM 40340T were grown for 10 days at 28 °C in tryptic soy broth medium with shaking at 150 r.p.m. and harvested and washed by repeated centrifugation in sterile water. Wet cells (100 mg) were saponified, methylated and the fatty acid methyl esters (FAMEs) analysed using GC–MS (Sasser, 2001). The GC–MS analysis was performed on a 7890A gas chromatograph (Agilent) directly coupled to the mass spectrometer system of a 5975C inert MSD (Agilent) with triple-axis detector. The MSD ChemStation was used to find all the peaks in the raw GC chromatogram. A library search was carried out for all the peaks using the NIST/EPA/NIH version 2.0.

The base composition of genomic DNA isolated from strain SUK12T was determined by a thermal denaturation method as described by Mandel & Marmur (1968), with Streptomyces coelicolor A3(2) as a control. The percentage G+C content was calculated as described by Owen & Pitcher (1985). The DNA was dissolved in 0.1 × standard saline citrate (SSC/10; SSC is 0.15 M NaCl/0.015 M trisodium citrate, pH 7.0). The DNA was quantitatively analysed and the melting temperature was determined with a Uvi Light XTD 5 (SECOMAM) spectrophotometer.

Genomic DNA was extracted and the 16S rRNA gene sequence amplified by PCR using universal bacterial 16S ribosomal DNA primers.
rRNA gene primers as described by Coombs & Franco (2003). The almost complete 16S rRNA gene sequence (1416 nt) was determined for strain SUK12<sup>T</sup>. The sequence was compared against the GenBank database using the BLAST program (Altschul et al., 1997) and EzTaxon-e database (Kim et al., 2012). Calculation of pairwise sequence similarity was done by using the global alignment algorithm, which was implemented at the EzTaxon server. The sequence was also multiply aligned with the 16S rRNA gene sequences of type strains of closely related species of the genus *Streptomyces* available from GenBank/EMBL using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic trees were reconstructed by using the neighbour-joining method (Fig. 1) of Saitou & Nei (1987), maximum-parsimony (Fig. S1 available in IJSEM Online) (Fitch, 1972) and maximum-likelihood (Fig. S2) (Felsenstein, 1981) tree-making methods available in the software package MEGA version 5 (Tamura et al., 2011). For the neighbour-joining and maximum-likelihood analyses, distance matrices were calculated according to Kimura’s two-parameter correction model (Kimura, 1980). Close-neighbour-interchange (search level=2, random addition=100) was applied in maximum-parsimony analysis. The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

DNA–DNA hybridization between strain SUK12<sup>T</sup> and *S. corchorusii* DSM 40340<sup>T</sup> which was used as probe, was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Identification Service. DNA in the crude lysate was purified by chromatography on hydroxyapatite (Cashion et al., 1977). DNA–DNA hybridization was carried out as reported by De Ley et al., (1970) with consideration of the modifications as described by Huss et al. (1983).

Strain SUK12<sup>T</sup> grew on a range of agar media (Table S1), showing morphology typical of streptomycetes. On ISP2 the colour of the substrate mycelium tended to be greenish-yellow and the aerial spore mass was greenish-grey. Aerial hyphae differentiated into Rectiflexibles chains of ellipsoidal spores (0.7–0.8 µm in length) with a spiny surface (Fig. 2).

The phenotypic profile of strain SUK12<sup>T</sup> is shown in Table S2. The results also showed that whole-cell hydrolysates of strain SUK12<sup>T</sup> contained LL-A<sub>2</sub>pm. The organism contained 63.6 % MK-9(H<sub>8</sub>) as the predominant isoprenoid in addition to 2.0 % MK-8(H<sub>8</sub>), 1.0 % MK-9(H<sub>2</sub>), 2.8 % MK-9(H<sub>4</sub>), 12.6 % MK-9(H<sub>6</sub>) and 18.0 % MK-9(H<sub>10</sub>) as shown in Fig. S3. The polar lipid profile of strain SUK12<sup>T</sup> consisted of the major compound diphostatidylglycerol

**Fig. 1.** Neighbour-joining tree showing the relationship of strain SUK12<sup>T</sup> based on nearly full 16S rRNA gene sequence (1416 nt) with closely related members of the genus *Streptomyces* and *Kitasatospora setae* KM-6054 as the outgroup. Asterisks indicate branches of the tree that were also recovered by using maximum-parsimony and maximum-likelihood algorithms. Numbers at the nodes indicate levels of bootstrap support based on 1000 resampled datasets. Bar, 0.01 changes per nucleotide.
and moderate amounts of phosphatidylinositol, phosphatidyethanolamine and phosphatidylglycerol as shown in Fig. S4. The profile corresponds to phospholipid type II (Lechevalier et al., 1977), and similar profiles have been reported for numerous species of the genus *Streptomyces*. The fatty acid profile detected in strain SUK12T was clearly different from that of *S. corchorusii* DSM 40340T (Table 1).

The G+C content of the DNA of strain SUK12T was 74 mol%.

An almost complete 16S rRNA gene sequence was determined for strain SUK12T (1416 nt). This sequence served to distinguish it from other streptomycetes by forming a distinct phyletic line from the most closely related species in both neighbour-joining and maximum-parsimony algorithms (Figs 1 and S2). It was evident from the phylogenic tree that strain SUK12T was closely related to the type strain *S. corchorusii* DSM 40340T (16S rRNA gene sequence similarity of 98.2 %) and *Streptomyces chrestomyceticus* NRRL B-3310T (16S rRNA gene sequence similarity of 98.1 %).

The level of DNA–DNA relatedness between strain SUK12T and its probe, *S. corchorusii* DSM 40340T was 18.85 ± 4.55 %. The definition of species in the genus *Streptomyces* recommends that DNA–DNA relatedness values below 80 % should constitute recognition of novel genomic species (Labeda, 1992). The low DNA–DNA relatedness value further confirms that strain SUK12T can be considered as a representative of a novel taxon.

The novel strain grew well on various ISP agar media, nutrient agar and PDA. The aerial spore mass of strain SUK12T was greenish-grey after 14 days of incubation at 28°C. The mycelia of the strain did not produce any melanoid pigment on tyrosine agar. The aerial mycelium of the strain was moderately short with simple branches and formed Rectiflexibles chains of spores on oatmeal agar at 14 days of culture. The spore surface of strain SUK12T was spiny (Fig. 2).

Phenotypic properties of the isolate were compared with those of the type strains, *S. corchorusii* NRBC 13032T, *S. chrestomyceticus* NRRL B-3310T, *Streptomyces olivaceoviridis* NBRC 13066T and *Streptomyces canarius* NBRC 13431T. These type strains of species of the genus *Streptomyces* were chosen as being the closest phylogenetic neighbours of the novel strain, sharing high 16S rRNA gene sequence similarity (98 % and above). Descriptions by Shirling & Gottlieb (1969, 1972) and Kato et al. (1993) were used to further differentiate SUK12T (Table 2). The results showed that strain SUK12T was clearly different from the representatives of the most closely related species. It is also clear that the organism forms a distinct phyletic line and also forms a different clade in the 16S rRNA gene sequence phylogenetic tree (Fig. 1).

The result showed that the G+C content of genomic DNA of this strain was 74 mol% and is within the range reported for other species of the genus *Streptomyces* (69–78 mol%) (Korn-Wendish & Kutzner, 1992). The DNA–DNA relatedness data together with phylogenetic distinctiveness were sufficient to categorize strain SUK 12T as distinct from previously recognized species in the genus *Streptomyces*.

From the above observations in the polyphasic taxonomy study, it is evident that strain SUK12T was clearly different from all other species of the genus *Streptomyces* with validly

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**Table 1. Major fatty acids (%) of strain SUK 12T and *S. corchorusii* DSM 40340T**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain 1</th>
<th>Strain 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12 : 0</td>
<td>18.5</td>
<td>–</td>
</tr>
<tr>
<td>C14 : 0</td>
<td>28.6</td>
<td>–</td>
</tr>
<tr>
<td>C15 : 0</td>
<td>13.6</td>
<td>7.0</td>
</tr>
<tr>
<td>C16 : 0</td>
<td>6.7</td>
<td>5.6</td>
</tr>
<tr>
<td>C17 : 0</td>
<td>–</td>
<td>0.3</td>
</tr>
<tr>
<td>C18 : 0</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>anteiso-C15 : 0</td>
<td>–</td>
<td>30.2</td>
</tr>
<tr>
<td>iso-C14 : 0</td>
<td>7.4</td>
<td>–</td>
</tr>
<tr>
<td>iso-C17 : 0</td>
<td>–</td>
<td>17.1</td>
</tr>
<tr>
<td>iso-C16 : 0</td>
<td>9.8</td>
<td>31.5</td>
</tr>
<tr>
<td>C18 : 1 n-9</td>
<td>–</td>
<td>1.9</td>
</tr>
<tr>
<td>C17 : 1 n-9</td>
<td>15.3</td>
<td>4.3</td>
</tr>
<tr>
<td>C18 : 1 n-7</td>
<td>–</td>
<td>1.6</td>
</tr>
<tr>
<td>iso-C18 : 1 n-11</td>
<td>–</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Scanning electron micrograph of strain SUK12T grown on ISP 2 for 14 days at 28 °C. Bar, 2 μm.
published names. For this reason, we propose the name *Streptomyces kebangsaanensis* sp. nov. to accommodate this strain.

**Description of Streptomyces kebangsaanensis** sp. nov.

*Streptomyces kebangsaanensis* (ke.bang.sa.a.nen'sis. N.L masc. adj. *kebangsaanensis*, pertaining to Universiti Kebangsaan Malaysia).

Gram-staining-positive, non-acid-fast, mesophilic actinobacterium. The organism forms branched substrate hyphae and differentiates into Rectiflexibles chains of ellipsoidal spores (0.7–0.8 μm in length) of six to ten spores. The spore surface is spiny. Aerial spore mass is greenish-grey and substrate mycelium is greenish-yellow on ISP 2 medium. No melanin is produced in tyrosine agar or tryptone-yeast extract broth. Able to degrade casein, xanthine and starch but unable to degrade tyrosine. Resistant to lysozyme at a concentration of 0.005 % (w/v) and to the antibiotics ampicillin, penicillin G, amphotericin B, meticillin, cycloheximide, oxacillin, nystatin and nalidixic acid. Able to utilize D-fructose, D-mannitol, sucrose, D-xylene, D-glucose, myo-inositol and rhamnose but not raffinose as sole carbon source for energy and growth. The peptidoglycan cell wall contains LL-3-diaminopimelic acid. The predominant menaquinone is MK-9(H8) followed by MK-8(H8), MK-9(H4), MK-9(H6) and MK-9(H10). The polar lipid profile of strain SUK12T consisted of the major compound diphosphatidylglycerol and moderate amounts of phosphatidylethanolamine and phosphatidylglycerol. The major fatty acid is C14:0.

The type strain SUK12T (= DSM 42048T = NRRL B-24860T) was isolated from the ethnomedicinal plant *Portulaca oleracea* L. collected from the Nenasi Reserve Forest, Pahang, Malaysia (2° 53.852’ N 103° 25.507’ E). The G+C content of its genomic DNA is 74 mol%. Affiliation of 16S rRNA gene sequence with *S. corchorusii* DSM 40340T is 98.2 %. DNA–DNA relatedness between strain SUK12T and *S. corchorusii* DSM 40340T is 18.85 ± 4.55 %.

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**References**


