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Actinopolymorpha singaporensis gen. nov., sp. nov., a novel actinomycete from the tropical rainforest of Singapore

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A novel actinomycete was isolated from soil in the tropical rainforest of Singapore. The cells of this actinomycete are highly pleomorphic. In the early stages of growth, most cells are of irregular squarish shape and varied sizes. Cells remain attached after cell division, often forming chains or aggregates of a few cells. Cells at the end of a chain tend to elongate. With prolonged cultivation, cells show different degrees of elongation and enlargement, producing branched hyphae of uneven thickness. At the periphery of the colony, long hyphae form, which are divided into alternating segments of elongated cells and chains of squarish cells. This actinomycete is considerably salt-tolerant, able to grow in the presence of 15% NaCl. Chemotaxonomically, it contains LL-diaminopimelic acid (DAP) in the cell wall, type PI phospholipids and MK-9(H_6) as the predominant menaquinone. 16S rDNA sequence analysis assigned this actinomycete to the family *Nocardioidaceae*, but its 16S rDNA shared no more than 91·2% sequence similarity with other members of the family. Based on phenotypic, chemotaxonomic and phylogenetic evidence, it is proposed that this actinomycete be classified as a new species in a new genus, *Actinopolymorpha singaporensis* gen. nov., sp. nov.

Keywords: *Actinopolymorpha singaporensis*, *Nocardioidaceae*, 16S rDNA, phylogeny, tropical rainforest

INTRODUCTION

The actinomycete family *Nocardioidaceae* was initially proposed by Nesterenko *et al*. (1985) to accommodate two genera, *Nocardioides* (Prauser, 1976) and *Pimelobacter* (Suzuki & Komagata, 1983). After the transfer of the *Pimelobacter* species to the genera *Terrabacter* and *Nocardioides* (Collins *et al*., 1989), *Nocardioides* became the only genus in the family. In a proposal of a new hierarchic classification system defined by sequence analyses of 16S rRNA or rDNA, Stackebrandt *et al*. (1997) included *Aeromicrobium* (Miller *et al*., 1991) in this family. Recently, a new genus, *Kribbella*, was proposed and assigned to this family on the basis of phylogenetic affiliation concluded from 16S rDNA sequence analysis (Park *et al*., 1999). In our previous investigation of the actinomycete diversity in the tropical rainforest of Singapore (Wang *et al*., 1999), we noticed that an LL-DAP-containing strain, IM 7744^T, formed a distinct branch within the clade of members of the family *Nocardioidaceae*, implying that it possibly belonged to a novel genus. To substantiate the result of the phylogenetic analysis, we carried out a detailed analysis of the morphological, physiological, biochemical and chemotaxonomic characteristics of strain IM 7744^T in addition to a more comprehensive 16S rDNA sequence comparison. We found that this actinomycete possesses novel properties that clearly distinguish it from all known actinomycetes. Here, we propose a new genus and a new species, *Actinopolymorpha singaporensis* gen. nov., sp. nov.

Abbreviations: DAP, diaminopimelic acid; TSB, tryptic soy broth.

The GenBank accession number for the 16S rDNA sequence of strain IM 7744^T is AF237815.

METHODS

Isolation. Strain IM 7744^T was isolated by the following procedure. Soil samples were collected from the surface to about 10 cm deep in the primary rainforest of the Bukit

Timah nature reserve in Singapore. The soil samples were dried in a chemical fume hood for 3 d and ground in a mortar. One milligram of the soil sample was first suspended in 10 ml Luria–Bertani (LB) medium containing 100 μg each of penicillin and streptomycin ml^{-1} for a 3 h incubation at 37 °C with vigorous shaking in order to kill fast-growing bacteria. This culture was centrifuged and washed three times with 10 ml sterile water and resuspended in 1 ml sterile water before being serially diluted and spread onto ISP 2 (International *Streptomyces* Project medium 2), ISP 3 and ISP 4 agar plates and incubated at 28–30 °C for 30 d. Recipes for the media used in this study are from the *Handbook of Microbiological Media* (Atlas, 1993) unless indicated otherwise.

Morphological characteristics. Strain IM 7744^T was grown on ISP 2, ISP 3, ISP 4 and Bennett's agar at 28 °C. For microscopic examination of cell morphology, cells were grown onto glass cover-slips inserted into the agar, as described previously (Hayakawa & Nomomura, 1993). Cells were examined under a light microscope at different times during growth and some samples were processed for examination with a scanning electron microscope (model JEOL JSM-5600LV) following the procedure described by Beveridge *et al.* (1994).

Physiological and biochemical characterization. In order to determine physiological and biochemical characteristics, we used the ISP media described by Shirling & Gottlieb (1966) and the media described by Waksman (1961). Cultures were incubated at 28 °C and properties were recorded after 7, 14, 21, 28 and 35 d. Culture colour was determined by referring to the colour chips from the *Color Harmony Manual* (Jacobson *et al.*, 1958). Carbohydrate utilization was determined by the method of Pridham & Gottlieb (1948). The temperature range for growth was determined on ISP 3 and ISP 4 media. Levels of tolerance of NaCl were determined on ISP 3 and tryptic soy broth (TSB) media. Gelatin liquefaction, starch hydrolysis and reaction on milk were tested by following the methods of Gordon *et al.* (1974) and Goodfellow (1971).

Chemotaxonomic characterization. The mycelia used for chemotaxonomic characterization of strain IM 7744^T were obtained as follows: a single colony was inoculated into a 250-ml flask containing 50 ml ISP 3 liquid medium and cultured at 28 °C for 2 weeks with vigorous shaking. Cells were harvested by centrifugation. Cell wall composition and the whole cell sugar pattern were determined as described by Lechevalier & Lechevalier (1965, 1980). Menaquinones were extracted by using the method of Collins *et al.* (1977) and analysed by HPLC, as described by Tamaoka *et al.* (1983). Polar lipids were extracted as described by Minnikin *et al.* (1979) and identified by two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980).

Preparation of genomic DNA. Genomic DNA was prepared as described previously (Wang *et al.*, 1996).

DNA base composition. The G+C content of the DNA was determined by the HPLC method (Mesbah *et al.*, 1989).

PCR amplification and sequencing of 16S rDNA. The oligonucleotide primers used for PCR amplification and sequencing of 16S rDNA were described previously (Wang *et al.*, 1996). Purified PCR products were sequenced by using an ABI automatic sequencer (model 377; Perkin-Elmer).

Database searching and phylogenetic analysis. Database searching was carried out by using the BLAST program. Multiple sequence alignment and computation of sequence similarities were conducted by using the CLUSTAL method of the DNASTAR program. Correct multiple sequence alignment was verified visually by using the consensus 16S rRNA secondary structure model as a reference (Gutell *et al.*, 1994). Phylogenetic trees were constructed by using both the neighbour-joining method of Saitou & Nei (1987) and the maximum-parsimony method (Swofford & Begle, 1993). The reliability of the trees was evaluated by using the bootstrap method of Felsenstein (1985).

RESULTS AND DISCUSSION

Isolation of slow-growing actinomycetes

We want to emphasize that preincubation of the soil suspension in a rich medium, such as LB medium, supplemented with penicillin and streptomycin is very effective in eliminating many fast-growing bacteria. Consequently, soil suspensions of high concentration can be spread onto isolation agar plates, greatly improving the chance of isolating slow-growing and rare actinomycetes. Diverse actinomycetes have been isolated by using this treatment (Wang *et al.*, 1999), which is exemplified by the identification of a novel actinomycete, strain IM 7744^T, described in this report.

Colonial and cultural characteristics

Strain IM 7744^T grows slowly on agar of all the media tested. The colonies are initially smooth and later become wrinkled. The organism grows mainly on the agar surface. Aerial hyphae are formed rarely and the vegetative hyphae weakly penetrate the agar surface only after prolonged cultivation on ISP 3 agar. The colour of the colonies is very yellow on ISP 3, ISP 4 and Bennett's agar plates and brilliant orange on ISP 2 plates. No diffusible pigment is produced on these agar plates. Growth occurs at 25 and 37 °C but not at 45 °C. The strain grows well in TSB containing 8 % NaCl. At 10–15 % NaCl, the cells can still grow, albeit much more slowly.

Cell morphology

The cell morphology of strain IM 7744^T is highly pleomorphic. Cells grown in different media develop drastically different shapes. Cells grown on Bennett's medium agar exhibit irregular shapes and varied sizes, some being triangular and some squarish. Cells appear to divide through budding and remain attached after division, often forming short chains and small aggregates (Fig. 1a). On ISP 4 agar, cells show greater size variations (Fig. 1b) and limited elongation. On ISP 3 agar, cells are more elongated and branches are formed (Fig. 1c). Cells show different degrees of elongation and enlargement, forming short branched hyphae of uneven thickness with shallow constrictions at intervals of varied length. The cells appear to grow by both apical and lateral budding followed by extension and swelling of certain segments. After 20 d cultivation, long hyphae that are divided into alternating segments of highly elongated cylindrical cells

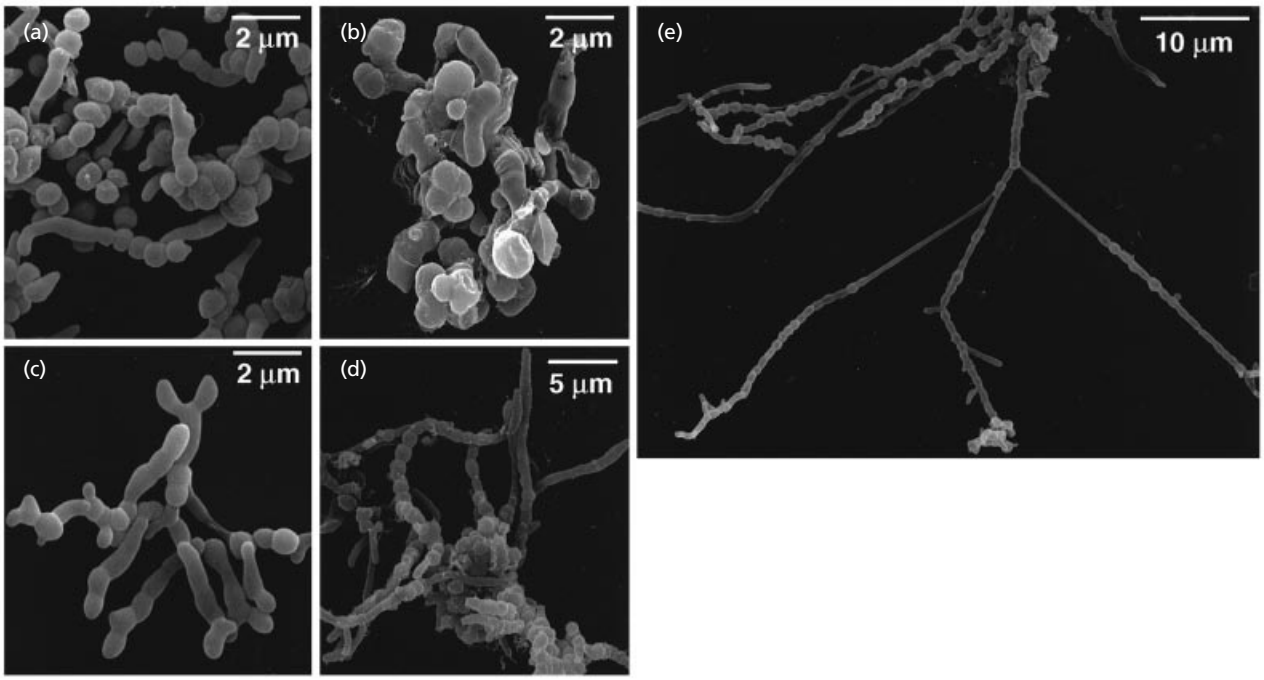

Fig. 1. Scanning electron micrographs of strain IM 7744^T. Strain IM 7744^T was grown for 8 d on agar plates of Bennett's (a), ISP 4 (b) and ISP 3 (c) media and for 25 d (d) and 35 d (e) on ISP 3 medium.

and chains of squarish cells form at the periphery of the colony (Fig. 1d, e), more prominently on ISP 3 than on other agar media. The hyphae may fragment, especially when the culture is moved to and maintained at 4 °C. No motile cells were found.

Physiological and biochemical characteristics

The physiological and biochemical characteristics of strain IM 7744^T are presented in the description of the new species.

Chemotaxonomic characteristics

The cell wall of strain IM 7744^T contains LL-DAP and glucose, rhamnose and ribose. MK-9(H_6) is the predominant isoprenoid quinone. MK-9(H_4), MK-9(H_8) and MK-10(H_4) are also present, but in smaller amounts. The phospholipid composition of strain IM 7744^T includes phosphatidylinositol mannosides, phosphatidylinositol, diphosphatidylglycerol and phosphatidylglycerol, but none of the diagnostic species phosphatidylethanolamine, phosphatidylcholine or unknown glucosamine-containing phospholipids. Thus, the phospholipid pattern of strain IM 7744^T corresponds to type PI (Lechevalier *et al*., 1977).

16S rDNA sequence analysis

In our previous study (Wang *et al*., 1999), a partial 16S rDNA sequence of ~ 900 bp was used in the phylogenetic analysis. Here, we obtained an almost complete 16S rDNA sequence comprising 1442 nucleotides (*Escherichia coli* numbering 38–1503) for strain IM 7744^T. The phylogenetic relationship of strain IM 7744^T to known actinomycete species was first estimated through a BLAST search of the GenBank database. In agreement with our previous result, strain IM 7744^T was found to share the highest 16S rDNA sequence similarity with members of the genera *Kribbella* and *Nocardioides*. For a more robust analysis, all the available 16S rDNA sequences from members of the family *Nocardioidaceae* and of all genera characterized by wall chemotype type I were chosen for pairwise sequence comparison and construction of phylogenetic trees.

Strain IM 7744^T shared the highest 16S rDNA sequence similarities of 90·6 and 91·2% with *Kribbella flavida* and *Kribbella sandramycini*, respectively, and less than 90% with all other actinomycetes. The generally low level of similarity indicates a distant relationship between strain IM 7744^T and known actinomycetes. The phylogenetic position of strain IM 7744^T was evaluated by constructing phylogenetic trees using both the distance-based neighbour-joining method of Saitou & Nei (1987) and the maximum-parsimony method of Swofford & Begle (1993). Both methods consistently placed strain IM 7744^T in the clade encompassing members of the family *Nocardioidaceae* with high bootstrap support. This phylogenetic placement was not affected by the choice of different outgroups. Fig. 2 shows part of a phylogenetic tree that included sequences of representative members of

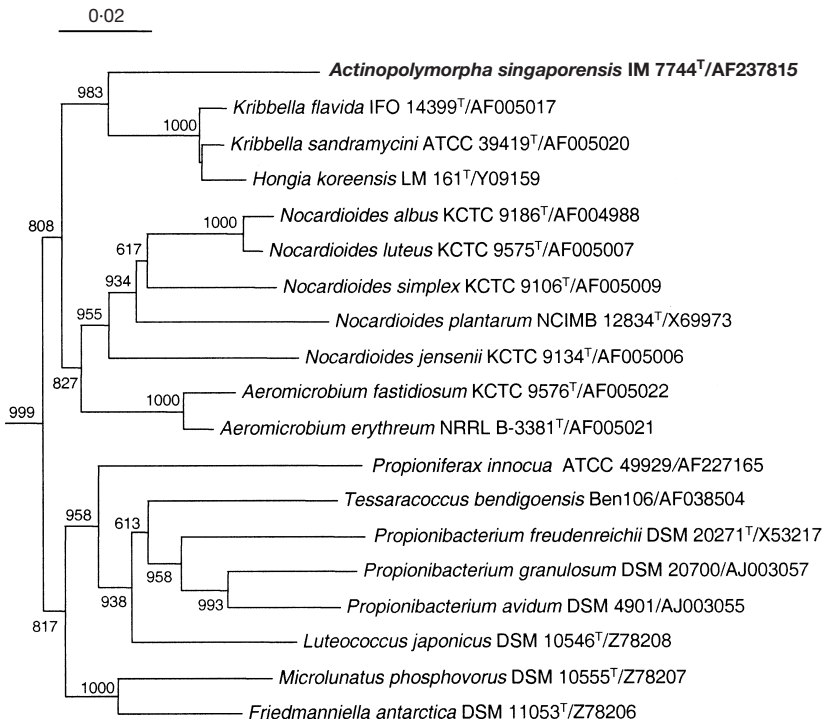

Fig. 2. A neighbour-joining phylogenetic tree of the suborder *Propionibacterineae*. This is part of a tree that included 16S rDNA sequences (nucleotides 38–1503) of representatives from all LL-DAP-containing genera. Inclusion or exclusion of alignment gaps had no effect on the position of strain IM 7744^T. Numbers at the nodes are the bootstrap values based on 1000 resamplings. The bar indicates the number of inferred substitutions per 100 nucleotides. The accession numbers for each strain and the 16S rDNA sequences are given.

Table 1 Signature 16S rDNA nucleotides in the *Nocardioidaceae*

Nucleotide signatures for the family are taken from Stackebrandt *et al.* (1997). Differences from the signature nucleotides of the *Nocardioidaceae* observed in strain IM 7744^T are shown in bold.

Nucleotide position(s)	Family *Nocardioidaceae*	IM 7744^T	*Kribbella*	*Hongia*
66:103	G:C	G:C	G:C	G:C
328	C	C	C	C
370:391	G:C	**C:G**	C:G	C:G
407:435	A:T	A:T	A:T	A:T
602:636	G:T	**A**:T	G:T	G:T
658:748	T:A	T:A	T:A	T:A
686	T	T	T	T
780	G	G	G	G
787	A	A	A	A
819	T	T	T	T
825:875	G:C	G:C	G:C	G:C
1409:1491	C:G	C:G	C:G	C:G

the suborder *Propionibacterineae*, which includes the family *Nocardioidaceae*, and all LL-DAP-containing genera. Within the family, strain IM 7744^T clustered with members of the genera *Kribbella* and *Hongia*, a recently proposed genus (Lee *et al.*, 2000), in a subclade that was distinct from a second subclade embracing *Nocardioides* and *Aeromicrobium* species. Both subclades were supported by high bootstrap values. Lee *et al.* (2000) did not resolve the family placement of *Hongia* when they proposed the genus. Our study

Table 2 Differential characteristics of strain IM 7744^T and related taxa containing LL-DAP

Taxon	Cell morphology	Major menaquinones	Polar lipids*	G+C content (mol%)	Reference
Strain IM 7744^T	Pleomorphism to hyphae	MK-9(H_4), MK-9(H_6), MK-9(H_8), MK-10(H_4)	PI, PIM, DPG, PG	69·5	This study
Kribbella	Hyphae	MK-9(H_4)	PC	70	Park *et al.* (1999)
Nocardioides	Hyphae, rods, cocci	MK-8(H_4)	PG, DPG, PL, PG-OH	66·5–71·7	Collins *et al.* (1989)
Aeromicrobium	Rods	MK-9(H_4)	PE, PG	71–73	Yokota *et al.* (1994)
Friedmanniella	Cocci in packets	MK-9(H_4)	PI, PG, DPG, PL	73	Schumann *et al.* (1997)
Microlunatus	Cocci	MK-9(H_4)	PI, PG, DPG, PL	67·9	Nakamura *et al.* (1995)
Luteococcus	Cocci	MK-9(H_4)	PI, PG, DPG, GL	66–68	Tamura *et al.* (1994)
Propioniferax	Rods	MK-9(H_4)	PE, PG, GL, PL	59–63	Yokota *et al.* (1994)
Propionibacterium propionicus	Pleomorphic rods	MK-9(H_4)	ND	63–65	Schumann *et al.* (1997)
Tessaracoccus	Cocci	MK-9(H_4), MK-7(H_4)	ND	74	Maszenan *et al.* (1999)
Intrasporangium	Hyphae	MK-8	PI, PIM, PG, DPG	68·2	Schumann *et al.* (1997)
Terrabacter	Rods, cocci	MK-8(H_4)	PE, PI, DPG, PL	69·8–73·4	Collins *et al.* (1989)
Terracoccus	Cocci	MK-8(H_4)	PE, PI, PG, DPG	73	Prauser *et al.* (1997)
Sporichthya	Hyphae	MK-9(H_6), MK-9(H_8)	PI, PG, DPG, PL	70·1	Rainey *et al.* (1993)
Streptomyces	Hyphae	MK-9(H_6), MK-9(H_8)	PE, PI, PIM, DPG	69–73	Williams *et al.* (1989)
Kineosporia†	Hyphae	MK-9(H_4)	PC	68–71	Kudo *et al.* (1998)

*DPG, diphosphatidylglycerol; GL, unknown glycolipid(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PG-OH, phosphatidylglycerol containing 2-hydroxy fatty acids; PI, phosphatidylinositol; PL, unknown phospholipid(s); PIM, phosphatidylinositol mannosides; ND, not determined.

† Species of this genus contain both LL-DAP and meso-DAP.

shows that *Hongia* has a very close phylogenetic relationship with *Kribbella* on the basis of 16S rDNA sequence analysis, suggesting that *Hongia* is a member of the family *Nocardioidaceae*. A check of the nucleotide signatures specific to the family *Nocardioidaceae* (Stackebrandt *et al.*, 1997) revealed the presence of most of the specific nucleotide signatures in the sequence of strain IM 7744^T, except for variations at a few positions (Table 1). The sequence of strain IM 7744^T has a C:G pair at nucleotide positions 370:379 instead of G:C and an A:T pair at 602:636 instead of G:T. Interestingly, the sequences of the members of the two newly proposed genera in this family, *Kribbella* and *Hongia*, also have a C:G pair at 370:379.

Conclusions

The results of sequence comparison and phylogenetic analysis suggest that strain IM 7744^T should be placed in the family *Nocardioidaceae*. Strain IM 7744^T possesses a number of properties that distinguish it from other established genera of the family, such as its unique morphology, its ability to grow in high NaCl concentrations, the presence of MK-9(H_6) as the predominant menaquinone, as opposed to MK-8(H_4) for *Nocardioides* and MK-9(H_4) for *Aeromicrobium*, *Kribbella* and *Hongia*, and its sharing of low levels of 16S rDNA sequence similarity with others. Table 2 summarizes the main differential characteristics of strain IM 7744^T and other LL-DAP-containing actinomycete taxa. In the 16S rDNA phylogenetic tree, strain IM 7744^T is separated from its closest neighbours, *Kribbella* and *Hongia*, by a long, distinct branch. Therefore, we believe that the polyphasic evidence presented above is sufficient for us to propose a new genus, *Actinopolymorpha* gen. nov., for strain IM 7744^T as *Actinopolymorpha singaporensis* sp. nov.

Description of *Actinopolymorpha* gen. nov.

Actinopolymorpha (Ac.ti.no.po.ly.mor′pha. Gr. n. *actis*, *actinos* a ray; Gr. adj. *poly* many; Gr. n. *morphus* form, shape; M.L. adj. *Actinopolymorpha* actinomycete of many shapes).

Slow growing. Irregular cells forming short chains or aggregates in the early stages of growth. Cells later exhibit varied degrees of elongation and swelling, forming branched hyphae with uneven thickness. Aerial mycelium absent or scarcely formed. Vegetative mycelia grow either on the agar surface in most media or penetrate poorly on ISP 3 medium at maturity. Colonies are pasty with yellow colour on ISP 3, ISP 4, TSA and Bennett's media and brilliant orange on ISP 2. Gram-positive. Strictly aerobic. Tolerates up to 15% NaCl in TSB liquid medium. The cell wall contains LL-diaminopimelic acid as the diamino acid in peptidoglycan. MK-9(H_6) is the predominant menaquinone; MK-9(H_4), MK-9(H_8) and MK-10(H_4) can also be detected. Glucose, rhamnose and ribose are

present in the whole cell hydrolysate. Phosphatidylinositol mannosides, phosphatidylinositol, diphosphatidylglycerol and phosphatidylglycerol are present (type PI). The genus is placed in the family *Nocardioidaceae* on the basis of 16S rDNA sequence analysis. All family-specific nucleotide signatures (Stackebrandt *et al.*, 1997) are present except for a C:G pair at nucleotide positions 370:379 instead of G:C and an A:U pair at positions 602:636 instead of a G:U pair. The type species is *Actinopolymorpha singaporensis* IM 7744^T (= JCM 10761^T = NRRL B-24113^T).

Description of *Actinopolymorpha singaporensis* sp. nov.

Actinopolymorpha singaporensis (sin.ga.po.ren′sis. *singaporensis* of Singapore, signifying the country where the type strain was isolated).

In addition to the characteristics described in the genus description, the actinomycete may use α-D-glucose, D-fructose, D-xylose, L-rhamnose, D-mannitol, sucrose, *myo*-inositol, sorbitol, D-galactose and glycerol as the sole carbon source, but not D-raffinose, D-arabinose or sodium succinate. Acid is produced from α-D-glucose, D-fructose, D-xylose, L-rhamnose, sucrose, sorbitol, *myo*-inositol and D-galactose. Positive for casein and gelatin hydrolysis, milk coagulation and peptonization and reduction of nitrate, but negative for starch degradation. Sensitive to 0·1 % phenol. Tolerant of up to 15% NaCl in TSB. The genomic DNA G+C content is 69·5 mol%. The type strain, IM 7744^T (= JCM 10761^T = NRRL B-24113^T), was isolated from a soil sample in the Bukit Timah nature reserve in Singapore.

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