Streptomyces koyangensis sp. nov., a novel actinomycete that produces 4-phenyl-3-butenoic acid

Jee Yeon Lee, Jung Yeop Lee, Ho Won Jung and Byung Kook Hwang

Laboratory of Molecular Plant Pathology, College of Life and Environmental Sciences, Korea University, Seoul 136-713, Korea

Correspondence
Byung Kook Hwang
bkhwang@korea.ac.kr

A 4-phenyl-3-butenoic acid-producing actinomycete, designated strain VK-A60^T, was isolated from a soil sample collected from Koyang, Korea. Morphological and chemical characteristics of the strain were consistent with those of the genus *Streptomyces*. The cell wall of the strain contains LL-diaminopimelic acid. The predominant fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{16:0}$. The strain formed a distinct monophyletic line within the 16S rRNA gene sequence phylogenetic tree. Analyses of its morphological, physiological and biochemical characteristics, together with random amplified polymorphic DNA and DNA–DNA relatedness data, confirmed that strain VK-A60^T represents a novel *Streptomyces* taxon that is distinguishable from closely related reference strains. Strain VK-A60^T (=KCCM 10555^T=NBRC 100598^T) is proposed as the type strain of a novel species, for which the name *Streptomyces koyangensis* sp. nov. is proposed.

The genus *Streptomyces* proposed by Waksman & Henrici (1943) for aerobic, spore-forming actinomycetes has been classified into different species based on morphology and cell-wall chemotaxonomic characteristics of the organisms. *Streptomyces* species, Gram-positive soil bacteria, have distinct features such as high DNA G+C content, the presence of LL-diaminopimelic acid (LL-DAP) and the absence of characteristic sugars in the cell wall (Anderson & Wellington, 2001). In addition, *Streptomyces* species produce extensively branched substrate and aerial mycelia (Locci, 1989). More than 500 *Streptomyces* species and subspecies have been described, the largest number of any bacterial genus (Hain *et al.*, 1997).

Streptomycetes are the most prolific producers of various bioactive compounds, such as antibiotics (Okami & Hotta, 1988; Berdy, 1995). During the screening of antifungal metabolites for plant chemotherapeutic agents, we isolated a variety of actinomycete strains that are active against some plant-pathogenic fungi and oomycetes from vegetative soil in Korea (Lee & Hwang, 2002). Among them, *Streptomyces* sp. strain VK-A60^T, isolated from soil in a radish field in Korea, produced the antibiotic 4-phenyl-3-butenoic acid, which exhibited antifungal activity against plant-pathogenic fungi *in vitro* and *in vivo* (Lee, 2002).

Here, strain VK-A60^T was subjected to a polyphasic taxonomic analysis. Based on morphological, physiological, phylogenetic and molecular evidence, strain VK-A60^T is considered to represent a novel species, *Streptomyces koyangensis* sp. nov.

Strain VK-A60^T, which produces 4-phenyl-3-butenoic acid (Lee, 2002), was isolated from soil collected from radish-growing fields at Koyang, Korea. The reference strains *Streptomyces canescens* KCCM 40569^T (=DSM 40001^T), *Streptomyces coelicolor* KCCM 40636^T (=DSM 40233^T), *Streptomyces felleus* KCCM 40499^T (=DSM 40130^T), *Streptomyces griseus* subsp. *griseus* KCCM 32410 (=IFO 12875), *Streptomyces limosus* KCCM 40500^T (=DSM 40131^T), *Streptomyces odorifer* KCCM 40694^T (=DSM 40347^T), *Streptomyces sampsonii* KCCM 40365^T (=ATCC 25495^T) and *Streptomyces somaliensis* KCCM 40354 (=DSM 40267) were obtained from the Korean Culture Center of Microorganisms (KCCM), Seoul, Korea. Strain VK-A60^T and the reference strains were grown at 28 °C on yeast extract/malt extract agar and stored in 15 % glycerol yeast extract/malt extract at −70 °C until used.

The morphology of the spore chain and the spore surface ornamentation of strain VK-A60^T were examined by light and scanning electron microscopy of 14-day-old cultures on inorganic salt/starch agar as described by Williams & Davies (1967). The morphological categorization suggested by Pridham *et al.* (1958) was employed using the section

Abbreviations: LL-DAP, LL-diaminopimelic acid; ISP, International *Streptomyces* Project; RAPD, random amplified polymorphic DNA; TBR, tree bisection–reconnection.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptomyces koyangensis* VK-A60^T is AY079156.

Detailed characteristics of the strains analysed in this study are available as supplementary material in IJSEM Online.

terminology of the ISP (International *Streptomyces* Project) (Shirling & Gottlieb, 1969). The cultural properties of strain VK-A60^T were evaluated according to the guidelines of the ISP as described by Shirling & Gottlieb (1966) and Locci (1989). Strain VK-A60^T was examined for physiological and biochemical features as described by Shirling & Gottlieb (1966), Williams *et al.* (1983) and Locci (1989). The isomers of DAP in the whole cell hydrolysates were analysed by TLC (Lee & Hwang, 2002). Cellular fatty acids were prepared and analysed using the method of Guckert *et al.* (1991). The DNA G+C content of strain VK-A60^T was determined using the thermal denaturation method of Marmur & Doty (1962). The T_m value was measured by UV spectroscopy (UV/Visible spectrophotometer, Pharmacia Biotech).

Genomic DNA of strain VK-A60^T was isolated as described by Pospiech & Neumann (1995). The 16S rRNA gene sequence was amplified using modified universal primers, fD1 (AGAGTTTGATCCTGGG) and rP2 (ACGGCTACCTTGTTACGACTT) (Weisburg *et al.*, 1991). The PCR products were ligated into the PCR2.1-TOPO vector (Invitrogen) and transformed into *Escherichia coli* TOP 10 (Invitrogen). The 16S rRNA gene sequence of strain VK-A60^T inserted into the plasmid vector was sequenced on an ABI310 automatic DNA sequencer (Applied Biosystems) using Big Dye terminator cycle sequencing ready reaction kits (PE Applied Biosystems). DNA sequence analysis was performed using the BLAST network services at the NCBI (Altschul *et al.*, 1997) and DNASTAR software program version 4.03 (DNASTAR, Inc.).

The nearly complete 16S rRNA gene sequence of strain VK-A60^T was aligned with other *Streptomyces* nucleotide sequences using the CLUSTAL W program version 1.7 (Thompson *et al.*, 1994). Phylogenetic analyses were performed according to the neighbour-joining method (Saitou & Nei, 1987) and the maximum-parsimony method (Fitch, 1971) using PAUP* version 4b10 (Swofford, 2002). Maximum-parsimony analysis was performed with the heuristic search option with random addition sequences, branch swapping by tree bisection–reconnection (TBR) and MAXTREES set at 100. Gaps were treated as missing data and all nucleotide substitutions were equally weighted and unordered. The consistency index and retention index were calculated for all parsimony trees (Kluge & Farris, 1969; Farris, 1989). Relative robustness of individual branches was estimated by bootstrapping, using 1000 replicates, with heuristic searches, branch swapping by TBR and MAXTREES set at 100. For neighbour-joining analysis, the data analysed by the Hasegawa–Kishino–Yano (HKY85) distance model (Hasegawa *et al.*, 1985) were used for the construction of the neighbour-joining tree. To determine the support for each clade, bootstrap analysis was performed with 1000 replications and MAXTREES set at 10. Trees were rooted using the TREEVIEW program, version 1.6.6.

PCR amplifications were carried out in 50 μL volumes for random amplified polymorphic DNA (RAPD) analysis. The PCR mixture contained 400 ng DNA, 0·125 μM $MgCl_2$, 0·25 μM dNTPs (Takara), 0·125 μM primer, 1·5 U *Taq* DNA polymerase (Takara) and the appropriate amount of 10× buffer (Takara). DMSO was added to the PCR mixture to give a concentration of 10 %. Amplification was performed in a DNA thermal cycler programmed for 4 min at 95 °C, 40 cycles of 40 s at 94 °C, 45 s at 38 °C and 90 s at 72 °C and a final extension for 5 min at 72 °C. The oligonucleotide primers AM50 (CAGGAAACAGCTATGAC), AM62 [GTTTCGGTGGTCAT(AT)GCGT(TAG)AGG], AM63 [CCT(CTA)ACGC(AT)ATGACCACGAAAC] (Mehling *et al.*, 1995) and 70-34 (GGACCGCTAG) (Roberts & Crawford, 2000) were synthesized by GenoTech Corp. After separation by agarose gel electrophoresis, the amplified fragments were visualized by staining with ethidium bromide solution and were photographed under UV light.

DNA–DNA hybridization between strain VK-A60^T and comparative strains *S. griseus* subsp. *griseus* IFO 12875, *S. canescens* DSM 40001^T, *S. coelicolor* DSM 40233^T, *S. sampsonii* ATCC 25495^T, *S. odorifer* DSM 40347^T, *S. limosus* DSM 40131^T, *S. felleus* DSM 40130^T and *S. somaliensis* DSM 40267 was performed according to the method of Chung *et al.* (1999).

Strain VK-A60^T produced *Rectiflexibiles* spore chains containing more than 10 spores per chain. The spores were spherical in shape and 1·2 μm in diameter with a smooth surface. A scanning electron micrograph of spore chains of strain VK-A60^T cultured on inorganic salts/starch agar is available as Supplementary Fig. A in IJSEM Online. Aerial mycelia proliferated well on most of the ISP culture media. The colour of the substrate mycelia was brown. Soluble pigments were produced. Strain VK-A60^T grew well on yeast extract/malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts/starch agar (ISP4), peptone/yeast extract iron agar (ISP6) and tyrosine agar (ISP7).

Whole-cell hydrolysates contained LL-DAP as a diagnostic diamino acid of the cell-wall peptidoglycan. The G+C content of the genomic DNA was 67·8 mol%. The fatty acid composition of strain VK-A60^T is shown in Supplementary Table A in IJSEM Online. The predominant cellular fatty acids were 12-methyltetradecanoic acid (anteiso-$C_{15:0}$), 14-methylpentadecanoic acid (iso-$C_{16:0}$) and hexadecanoic acid ($C_{16:0}$). The morphological and chemical properties of strain VK-A60^T were consistent with those of the genus *Streptomyces* (Locci, 1989; Manfio *et al.*, 1995) (Table 1). In particular, strain VK-A60^T produced the antifungal compound 4-phenyl-3-butenoic acid in cell cultures (Lee, 2002).

The nearly complete 16S rRNA gene sequence (1488 nt) of strain VK-A60^T was compared to that of *Streptomyces* species deposited in the GenBank database. In the phylogenetic tree of 29 *Streptomyces* species, strain VK-A60^T was placed in the clade with *S. griseus* subsp. *griseus*, *S. felleus*, *S. odorifer*, *S. coelicolor*, *Streptomyces albidoflavus*, *S. somaliensis*, *S. limosus*, *S. canescens*, *S. sampsonii*, *Streptomyces resistomycificus* and *Streptomyces griseochromogenes* (Fig. 1). 16S

Table 1. Morphological, physiological and biochemical characteristics of strain VK-A60^T and phylogenetically related *Streptomyces* species

Strains/species: 1, *S. koyangensis* sp. nov. VK-A60^T; 2, *S. albidoflavus* 3, *S. canescens*; 4, *S. coelicolor*; 5, *S. felleus*; 6, *S. griseus*; 7, *S. limosus*; 8, *S. odorifer*; 9, *S. sampsonii*; 10, *S. somaliensis*. Data for reference species were taken from Shirling & Gottlieb (1966, 1969), Nonomura (1974) and Williams *et al.* (1989). +, Positive; −, negative; D, doubtful; V, variable; ND, no data available. All species exhibit *Rectiflexibiles* spore chains with smooth spore surfaces and are negative for growth on inositol and raffinose and positive for growth on xylose and mannitol as sole carbon sources.

Characteristic	1	2	3	4	5	6	7	8*	9	10
DNA G+C content (mol%)	67·8	70	ND	ND	ND	71	ND	ND	ND	ND
Aerial mass colour†	W/Y	W/G	Y	Y	Y	Y	Y	Y	Y/G	PY
Spores per chain (*n*)	Generally 10–50 or more	Generally short; 3–10	10–50	10–50, or often more	10–50 or more	10–50	10–50 or more	10–50 or more	Generally long; more than 50	10–50 or more
Melanin production	+	−	−	−	−	+/−‡	−	−	−	−
Soluble pigment	+	−	−	+	−	−	−	+	−	+
Reverse-side pigment on:§										
ISP2	B	−	−	LB/SB	−	−	−	−	−	−
ISP3	B	−	−	B	−	−	−	−	Y/YB	−
ISP4	B	−	GY	LB/SB	−	−	−	−	Y/YB	−
ISP5	C/LB	−	−	LB/SB	−	−	−	−	−	−
Growth on sole carbon sources:										
Arabinose	+	+	D/V	+	+	−	+	+	+	−
Fructose	+	+	D/V	+	+	+	+	+	+	+
Rhamnose	−	−	−	−	−	−	−	+/−	−	−
Sucrose	−	−	−	−	−	−	−	−/+	−	−
D-Glucose	+	+	+	+	V	+	+	+	+	+
Antibiotic produced‖	4-Phenyl-3-butenoic acid	ND	Asconsin	ND	Pikromycin	DAO	Limocrocin	ND	ND	ND

*S. *odorifer* strain CBS differs from strain IMRU in melanin production, carbon utilization, darkening of PYIA (peptone/yeast/iron agar) and TYB (tryptone yeast broth), but not TA (tyrosine agar). Strain CBS utilizes rhamnose, but not sucrose, whereas strain IMRU uses sucrose, but not rhamnose.

†G, Grey; PY, pale yellow; W, white; Y, yellow.

‡Melanin pigment is produced in ISP7 medium, but not in ISP6.

§B, Brown; C, creamy; GY, greenish yellow; LB, light brown; SB, strong, dark brown; Y, yellow; YB, yellowish brown.

‖DAO, Deacetoxycephalosporin.

rRNA gene sequence similarity among these organisms was 99 %, which corresponds to approximately 15 nt differences in 1488 bases. These 11 *Streptomyces* species were grouped into the clusters of *S. griseus* subsp. *griseus* and *S. albidoflavus* in the phylogenetic tree generated using the neighbour-joining algorithm (Saitou & Nei, 1987) (Fig. 1). The close relationships between strain VK-A60^T and these 11 species were confirmed by maximum-parsimony methods (Fitch, 1971). Strain VK-A60^T forms a distinct phylogenic line distant from the two clusters of *S. griseus* subsp. *griseus* and *S. albidoflavus* in the tree. The position of strain VK-A60^T in the phylogenetic tree was not affected by either the tree-making algorithm or the outgroup strains used. These findings suggest that strain VK-A60^T represents a novel species that is closely related to the 11 *Streptomyces* species, having a high 16S rRNA gene sequence similarity. The designation of strain VK-A60^T as a separate genomic species was suggested by the bootstrap value (96 %) in the neighbour-joining tree based on the nearly complete 16S rRNA gene sequence data.

To establish further the classification of strain VK-A60^T, RAPD analysis was performed using primers of various lengths with varying G+C contents. RAPD analysis yielded 6–13 bands for each *Streptomyces* strain and specific banding patterns with the different primer pairs. In general, primers AM50, AM62 and AM63 separated nine strains into three groups according to their banding patterns. In the RAPD profile, *S. griseus* subsp. *griseus* formed one group (group I), and *S. canescens*, *S. coelicolor*, *S. felleus*, *S. limosus*, *S. odorifer*, *S. somaliensis* and *S. sampsonii* formed another (group II). Strain VK-A60^T, in group III, exhibited a clearly distinguishable RAPD banding pattern. In particular, unique bands of 0·4, 3·0 and 0·9 kb were generated from strain VK-A60^T by the primers AM50, AM62 and AM63, respectively. When the primer 70-34 (with low

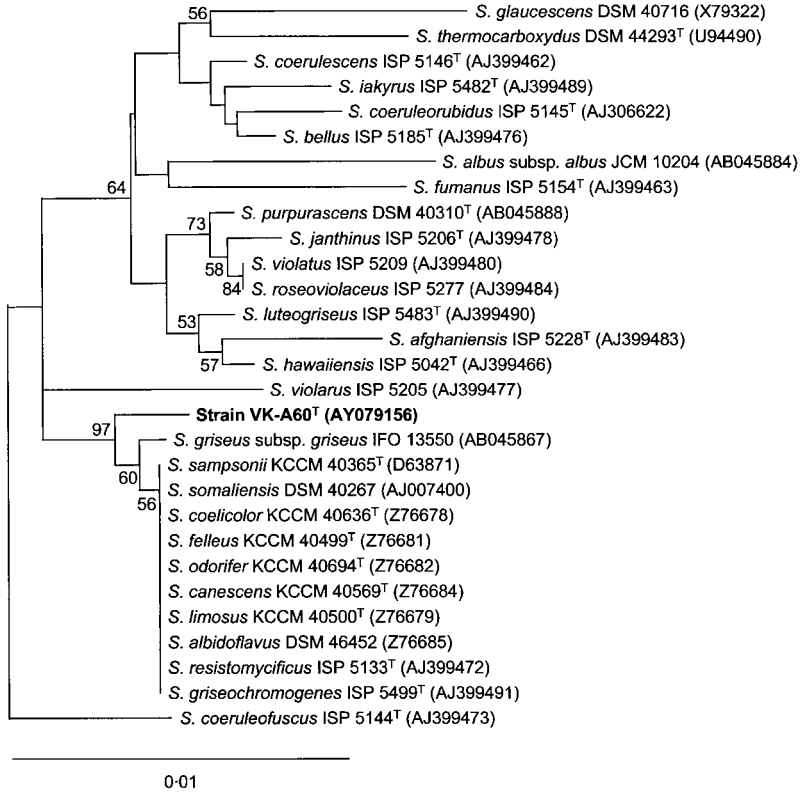

Fig. 1. Neighbour-joining phylogenetic tree of strain VK-A60^T and 28 *Streptomyces* species based on nearly complete 16S rRNA gene sequences (1488 nt). Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values >50 % are given. NCBI accession numbers are given in parentheses. Bar, 0·01 nucleotide substitutions per site.

DNA G+C content) was used, all strains tested generated diverse RAPD banding patterns. The RAPD banding patterns are available as Supplementary Fig. B in IJSEM Online.

DNA–DNA hybridization analysis was carried out between strain VK-A60^T and closely related strains selected from the phylogenetic data. The levels of DNA–DNA hybridization between strain VK-A60^T and *S. griseus* subsp. *griseus*, *S. canescens*, *S. coelicolor*, *S. sampsonii*, *S. odorifer*, *S. limosus*, *S. felleus* and *S. somaliensis* were 68·5, 55·2, 20·8, 64·0, 34·4, 66·8, 25·6 and 57·5 %, respectively. DNA–DNA relatedness values below 80 % have been recommended for the recognition of novel genomic species of *Streptomyces* (Labeda, 1993, 1996, 1998). The DNA–DNA relatedness values determined here are available as Supplementary Table B in IJSEM Online.

Strain VK-A60^T was clearly distinguished from the phylogenetically related *Streptomyces* strains by the formation of melanin, soluble pigments, reverse-side pigments and production of antibiotics (Table 1). However, morphological features of the aerial mycelium and carbon-source utilization of strain VK-A60^T were similar to those of these closely related strains.

Based on the combination of phenotypic characteristics and genotypic data, we propose the name *Streptomyces koyangensis* sp. nov. for this organism.

Description of *Streptomyces koyangensis* sp. nov.

Streptomyces koyangensis (ko.yang.en′sis. N.L. masc. adj. *koyangensis* pertaining to Koyang, Republic of Korea, the geographical origin of the type strain).

Aerobic, Gram-positive, non-motile actinomycete. Spore chains containing 10 or more spores per chain are *Rectiflexibiles*. Spores are spherical (1·2 μm in diameter) with a smooth surface. Whole-cell hydrolysates contain LL-DAP. The G+C content of the genomic DNA is 67·8 mol%. The predominant cellular fatty acids are anteiso-$C_{15:0}$ (16·54 %), iso-$C_{16:0}$ (28·77 %) and $C_{16:0}$ (11·60 %). In addition, anteiso-$C_{17:0}$ (9·01 %), iso-$C_{14:0}$ (8·84 %), iso-$C_{15:0}$ (7·02 %), $C_{17:0}$ cyclo (4·54 %), anteiso-$C_{17:1}$ (3·23 %), iso-$C_{17:0}$ (1·94), $C_{14:0}$ (1·33 %), iso-$C_{16:1}$ (1·86 %) and $C_{16:1}$ *cis*9 (2·57) were detected. Grows well on yeast extract/malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts/starch agar (ISP4), peptone/yeast extract iron agar (ISP6) and tyrosine agar (ISP7). Does not grow well on ISP5 medium. The spore mass is white to grey and the reverse sides of colonies are brown on most agar media. Aerial mycelia are abundant on most of these media. The colour of substrate mycelium is pale brown to dark brown. Production of spores on ISP4 is prolific. Melanin pigments are produced on ISP6 and ISP7. As the sole carbon source, it utilizes L-arabinose, D-fructose, mannitol and xylose for growth, but not adonitol, dextran,

meso-inositol, D-melezitose, D-melibiose, raffinose, L-rhamnose, sucrose or xylitol. As a nitrogen source, utilizes L-cysteine, L-histidine, L-phenylalanine and L-valine. However, it cannot utilize DL-α-amino-*n*-butyric acid or L-hydroxyproline. Degrades casein, elastin, aesculin, gelatin, starch, tyrosine and xanthine, but not cellulose. Pectin hydrolysis, nitrate reduction and H_2S production are positive, whereas lecithinase, lipolysis and hippurate hydrolysis are negative. The strain grows in the presence of 4, 7 and 10 % sodium chloride but not 13 %. It grows in 0·02 % NaN_3 and 0·001 % thallous acetate, but not in 0·1 % phenol or 0·001 % potassium tellurite. The strain is resistant to penicillin G, but sensitive to neomycin, rifampicin and oleandomycin. Produces 4-phenyl-3-butenoic acid, which inhibits the mycelial growth of several plant-pathogenic fungi, such as *Alternaria mali*, *Cladosporium cucumerinum*, *Colletotrichum gloeosporioides*, *Colletotrichum orbiculare*, *Magnaporthe grisea* and *Fusarium oxysporum* f. sp. *cucumerinum*.

The type strain is VK-A60^T (=KCCM 10555^T=NBRC 100598^T).

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