

## *Streptomyces cheonanensis* sp. nov., a novel streptomycete with antifungal activity

Hyo Jin Kim, Sung Chul Lee and Byung Kook Hwang

Correspondence  
Byung Kook Hwang  
bkhwang@korea.ac.kr

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

Actinomycete strain VC-A46<sup>T</sup>, which exhibits antifungal activity, was isolated from a soil sample collected from Cheonan, Korea. The strain was determined to belong to the genus *Streptomyces*, based on its morphological and chemotaxonomic characteristics. The cell wall of this strain contained LL-diaminopimelic acid. The predominant fatty acids were iso-C<sub>16:0</sub>, C<sub>16:0</sub> and C<sub>16:1 cis 9</sub>. The DNA G + C content of strain VC-A46<sup>T</sup> was 75.5 mol%. Phylogenetic analysis of the 16S rRNA gene sequence of this strain revealed that it differed from those of the described streptomycetes available in public databases. Analyses of DNA–DNA relatedness data, together with data regarding the strain's morphological, physiological and biochemical characteristics, also confirmed that this strain constitutes a novel *Streptomyces* taxon, which is distinguishable from closely related reference strains. Therefore, it is proposed that strain VC-A46<sup>T</sup> (=KCCM 42119<sup>T</sup>=NBRC 100940<sup>T</sup>) represents the type strain of a novel species, named *Streptomyces cheonanensis* sp. nov.

The genus *Streptomyces* was initially introduced by Waksman & Henrici (1943) as a term to encompass aerobic, spore-forming actinomycetes. Streptomycetes are used extensively in industry, due to their ability to generate a number of chemical compounds, including antibiotics, enzymes, enzyme inhibitors, antitumour agents and antifungal compounds (Chun *et al.*, 1997; Kim & Hwang, 2003). In particular, *Streptomyces* species are prolific producers of structurally diverse antibiotics. Most of the antibiotics developed for agricultural uses, including pesticides, were isolated from *Streptomyces* strains (Tanaka & Ômura, 1993). Among antifungal antibiotics discovered in recent years from *Streptomyces* strains, polyketide-spiroketal spirofungins, macrolide cineromycins, oligomycin A, antimycin-type kitamycins, aflatoxin inhibitor aflastatins, aminoacetophenone family heptaene antibiotics and novel nikkomycin analogues were found to have potent activity (Kim & Hwang, 2003).

In the present study, we isolated strain VC-A46<sup>T</sup> from the soil of a radish field in Cheonan, Korea (Lee & Hwang,

2002). Strain VC-A46<sup>T</sup> exhibited antifungal activity against the plant-pathogenic fungi *Alternaria mali*, *Colletotrichum orbiculare*, *Magnaporthe grisea*, *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* and antioomycete activity against *Phytophthora capsici* (data not shown). Morphological, physiological, biochemical, phylogenetic and molecular evidence was obtained and analysed in our identification of strain VC-A46<sup>T</sup>.

The soil suspension from a soil sample from Cheonan was seeded on a humic acid/vitamin agar medium and was subsequently incubated for 14 days at 28 °C. The reference strains *Streptomyces thermolineatus* KCCM 41146<sup>T</sup>, *Streptomyces macrosporus* KCCM 41250<sup>T</sup>, '*Streptomyces cattleya*' KCCM 11825, *Streptomyces acidiscabies* KCCM 41240<sup>T</sup> and *Streptomyces megasporus* KCCM 41251<sup>T</sup> were provided from the Korean Culture Center of Microorganisms, Seoul, Korea. Strain VC-A46<sup>T</sup> and the reference strains were grown on a yeast extract/malt extract agar medium (4 g yeast extract, 10 g malt extract, 4 g glucose, 15 g agar, 1 l water) at 28 °C and stored in 15% glycerol at –70 °C.

The morphological characteristics of strain VC-A46<sup>T</sup>, including spore chain morphology, spore size and surface ornamentation, were assessed by light and scanning electron microscopy (SEM) of 14-day-old cultures on yeast extract/malt extract (ISP 2) agar (Williams & Davies, 1967). Aerial spore-mass colour, substrate mycelium pigmentation and colouration of the diffusible pigments of strain VC-A46<sup>T</sup> were recorded on ISP (International *Streptomyces* Project) media (Shirling & Gottlieb, 1966) and as described by Locci

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Abbreviations: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *S. cheonanensis* VC-A46<sup>T</sup> is AY822606.

A scanning electron micrograph, a  $\gamma$ -region-based neighbour-joining tree and details of the fatty acid content analysis and DNA–DNA hybridization experiments are available as supplementary material in IJSEM Online.

(1989). The biochemical and physiological characteristics of strain VC-A46<sup>T</sup> were determined according to the methods described by Shirling & Gottlieb (1966), Williams *et al.* (1983) and Locci (1989).

For chemical analyses, biomass from strain VC-A46<sup>T</sup> was prepared by culturing in yeast extract/malt extract broth (4 g yeast extract, 10 g malt extract, 4 g glucose, 1 l water) for 4 days at 28 °C in a rotary shaker (120 r.p.m.) and harvested by centrifugation. The harvested cells were washed with methanol and water. Freeze-dried cells (20 mg) were then hydrolysed with 5 ml 6 M HCl at 100 °C for 18 h. The filtrate was evaporated to remove the remaining HCl. The isomer type of the diaminopimelic acid in the peptidoglycan layer was determined by TLC (Lee *et al.*, 2005), using a modified solvent system of methanol/water/10 M HCl/pyridine (80:26:2.5:20, by vol.) (Becker *et al.*, 1965), followed by ninhydrin staining (0.1% w/v ninhydrin in acetone). Cellular fatty acids were then prepared and analysed according to the method described by Guckert *et al.* (1991).

We analysed the G + C content of strain VC-A46<sup>T</sup> using the thermal melting method pioneered by Marmur & Doty (1962), Mandel *et al.* (1970) and Johnson (1985). Strain VC-A46<sup>T</sup> was inoculated into yeast extract/malt extract broth, followed by DNA extraction (150–250 µg ml<sup>-1</sup>). A 1/10 volume of 8 M LiCl and 2 vols ethanol were then added to the DNA extracts. After five washes with 70% ethanol, the remaining DNA was dissolved in 500 µl 0.1 × SSC. The DNA was quantitatively analysed using a spectrophotometer (DU-650; Beckman) and the melting temperature was measured with a UV/visible spectrophotometer (Ultraspec 2000; Pharmacia Biotech).

Strain VC-A46<sup>T</sup> was cultured on ISP 2 agar for 4 days at 28 °C and picked colonies were then cultured in LB broth (10 g NaCl, 10 g tryptone and 5 g yeast extract in 1 l water) in a rotary shaker for 2 days at 28 °C. Genomic DNA was isolated from cultured cells according to the method described by Pospiech & Neumann (1995). The 16S rRNA gene of strain VC-A46<sup>T</sup> was then amplified by PCR, using two universal bacterial primers, FD1 (5'-AGAGTTT-GATCCTGGG-3') and RP2 (5'-ACGGCTACCTTGTTA-CGACTT-3') (Weisburg *et al.*, 1991). These primer-based PCR amplifications were conducted on a PTC-200 thermal cycler (MJ Research). The samples were subjected to an initial denaturing step, for 4 min at 94 °C. The thermal profile comprised 25 cycles, consisting of 1 min denaturation at 98 °C, 1 min annealing at 57 °C and 2 min extension at 72 °C. Finally, a 3 min extension step at 72 °C and a final cooling to 4 °C completed the reaction sequence. PCR products were purified from 1% agarose gel (Wu *et al.*, 1997), ligated into the pCR 2.1-TOPO T vector (Invitrogen) and transformed into *Escherichia coli* TOP10 cells (Invitrogen) by electroporation. The purified PCR products were then sequenced on an ABI 310 automatic DNA sequencer (Applied Biosystems) using Big Dye terminator cycle sequencing ready reaction kits (PE Applied

Biosystems). 16S rRNA gene sequence analysis was then conducted using the BLAST network services provided by the NCBI (Altschul *et al.*, 1997) and the DNASTAR program, version 4.0 (DNASTAR). These sequences were also compared to the public nucleotide databases, using the BLAST algorithm, to identify 16S rRNA gene sequences with high degrees of similarity. The almost-complete 16S rRNA gene sequence of strain VC-A46<sup>T</sup> was aligned with representative sequences of related streptomycete species in the GenBank database (NCBI). Sequences were edited with the DNASTAR (Lasergene system for sequence analysis) computer package. Alignment was performed with the CLUSTAL W program (Thompson *et al.*, 1994). PAUP version 4.0b10 (Swofford, 2002) software was used to carry out the phylogenetic analyses. The resultant data were then examined using the maximum-parsimony method (Fitch, 1971) and the neighbour-joining method (Saitou & Nei, 1987). The topology of the resultant unrooted tree was evaluated by bootstrap assay of the neighbour-joining tree, performed in 1000 replications. Phylogenetic tree display, editing and printing were carried out using the TreeView program, version 1.6.6 (Page, 1996).

DNA–DNA hybridization experiments with strain VC-A46<sup>T</sup> and the reference strains *S. thermolineatus* DSM 41451<sup>T</sup>, *S. macrosporus* DSM 41449<sup>T</sup>, '*S. cattleya*' JCM 4925 and *S. acidiscabies* ATCC 49003<sup>T</sup> were performed according to the methods described by Chung *et al.* (1999). DNA probes were labelled with a Dig-High prime kit (Boehringer Mannheim). Hybridization was then performed on nylon membranes (Hybond-N<sup>+</sup>; Amersham Pharmacia Biotech).

Strain VC-A46<sup>T</sup> was observed to grow well on a variety of ISP agar media, including ISP 2, oatmeal agar (ISP 3), inorganic salts/starch agar (ISP 4), peptone/yeast extract agar (ISP 6) and tyrosine agar (ISP 7) (Shirling & Gottlieb, 1966). Aerial mycelium of strain VC-A46<sup>T</sup> was abundant on ISP 3 agar. The aerial mycelium was grey to white in colour and the substrate mycelium was light yellow on ISP 4. Diffusible pigments and melanin were generated on most ISP media. A scanning electron micrograph of the spore chains of strain VC-A46<sup>T</sup> can be viewed as Supplementary Fig. S1 in IJSEM Online. Strain VC-A46<sup>T</sup> is characterized by short or long spore chains with soft surfaces. Spores were elliptical in shape and 0.8 µm in length. The morphological, physiological and biochemical characteristics of strain VC-A46<sup>T</sup> and phylogenetically related *Streptomyces* species are shown in Table 1.

The DNA G + C content of strain VC-A46<sup>T</sup> was 75.5 mol%. Strain VC-A46<sup>T</sup> was also found to contain LL-diaminopimelic acid in the peptidoglycan of its cell walls, thereby indicating that strain VC-A46<sup>T</sup> exhibits cell-wall chemotype I (Lechevalier & Lechevalier, 1970). The fatty acid composition of strain VC-A46<sup>T</sup> is shown in Supplementary Table S1 in IJSEM Online. The predominant cellular fatty acids found included 14-methylpentadecanoic acid (iso-C<sub>16:0</sub>), hexadecanoic acid (C<sub>16:0</sub>) and *cis*-9-hexadecenoic acid (C<sub>16:1 cis 9</sub>).

**Table 1.** Morphological, physiological and biochemical characteristics of strain VC-A46<sup>T</sup> and phylogenetically related *Streptomyces* species

Strains: 1, VC-A46<sup>T</sup>; 2, *S. thermolineatus* DSM 41451<sup>T</sup>; 3, '*S. cattleya*' JCM 4925; 4, *S. macrosporus* DSM 41449<sup>T</sup>; 5, *S. megasporus* DSM 41476<sup>T</sup>. None of the strains grow at 4 °C. All of the strains degrade gelatin, are susceptible to neomycin (50 µg ml<sup>-1</sup>) and rifampicin (50 µg ml<sup>-1</sup>), grow on L-cysteine, L-histidine and L-valine (0.1%, w/v) as sole nitrogen sources and grow on fructose, L-rhamnose and xylose (1%, w/v) as sole carbon sources. +, Positive; -, negative; ?, result uncertain; ND, no data.

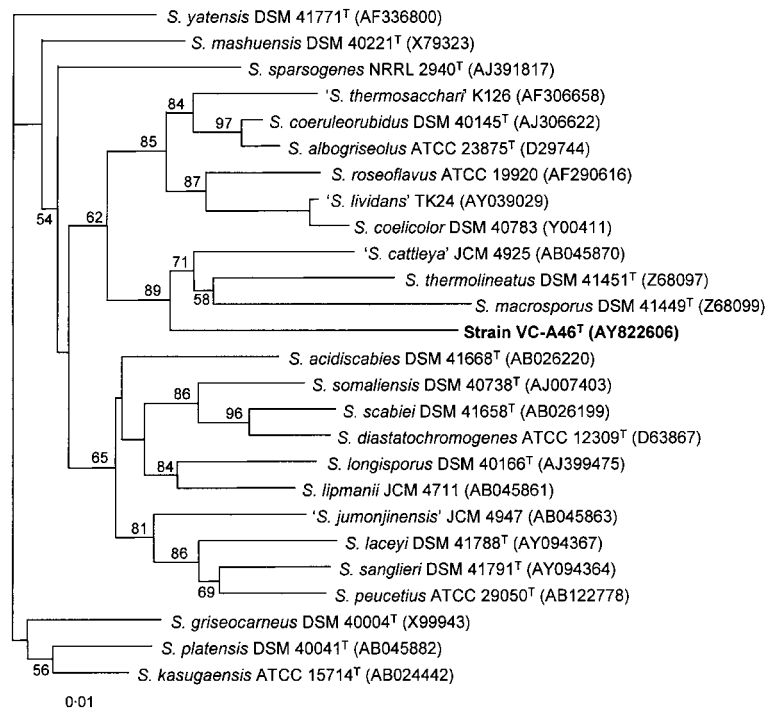
Characteristic	1	2	3	4	5
Spore chain morphology*	RF	RF	SP	RF	ND
Aerial hyphae	+	+	+	-	+
Melanin production	+	-	-	-	-
Maximum NaCl tolerance (% w/v)	7	6	3	6	ND
Colour of spore mass/substrate mycelium on:†					
ISP medium 2	PY/PY	WG/PY	OP/PY	None/PY	W/PY
ISP medium 3	G/O	W/W	OP/PY	None/PY	W/OW
ISP medium 4	GW/PY	WG/PY	OS/PY	ND	W/OW
ISP medium 5	O/O	WG/PY	OS/PY	ND	W/PY
ISP medium 6	GW/VY	W/PY	None/PY	ND	W/PY
ISP medium 7	GW/W	WG/PY	OS/PY	ND	GW/OW
Colour of soluble pigment on:					
ISP medium 3	DG	None	None	None	None
ISP medium 5	O	None	None	ND	None
ISP medium 6	None	None	SB	ND	SB
ISP medium 7	BW	None	None	ND	G
Degradation of:					
Aesculin	+	-	-	+	-
Starch	+	+	-	+	+
Resistance to oleandomycin (100 µg ml <sup>-1</sup> )	-	-	-	-	+
Growth at/in:					
45 °C	-	+	+	+	+
pH 5	-	-	+	-	-
pH 11	-	+	+	+	-
Growth on DL-α-amino-n-butyric acid as sole nitrogen source (0.1%, w/v)	+	+	+	+	-
Growth on sole carbon sources (1%, w/v):					
Adonitol	+	+	+	+	?
Raffinose	+	-	-	-	-
Antibiosis against:					
<i>Bacillus subtilis</i> NCIMB 3610 <sup>T</sup>	+	+	-	-	-
<i>Micrococcus luteus</i> NCIMB 196	+	+	+	+	-
<i>Saccharomyces cerevisiae</i> CBS 1171	+	-	+	+	-
<i>Streptomyces murinus</i> ISP 5091 <sup>T</sup>	+	-	+	+	-

\*RF, *Rectiflexibiles*; SP, *Spirales*.

†BW, Brown to white; DG, dark grey; G, grey; GW, grey to white; O, olive; OP, orchid mixed with pinkish white; OS, orchid mixed with some white; OW, oyster white; PY, pale yellow; SB, slight browning; VY, violet to yellow; W, white; WG, white to grey.

Comparison of the nearly complete 16S rRNA gene sequence (1527 nt) of strain VC-A46<sup>T</sup> with previously obtained sequences of *Streptomyces* species deposited in GenBank (NCBI) indicated that this organism is related phylogenetically to members of the genus *Streptomyces*. The rooted phylogenetic tree (Fig. 1) based on the

neighbour-joining method indicated that strain VC-A46<sup>T</sup> formed a distinct branch with the proposed type strain of '*S. cattleya*' and the type strains of *S. thermolineatus* and *S. macrosporus*. Strain VC-A46<sup>T</sup> exhibited 95.9% similarity to '*S. cattleya*' JCM 4925 (56 nucleotide differences at 1354 sites), 95.4% (62/1354) similarity to *S. thermolineatus* DSM



**Fig. 1.** Phylogenetic tree showing the relationship between strain VC-A46<sup>T</sup> and representative species of the genus *Streptomyces* based on nearly complete 16S rRNA gene sequences. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis (Saitou & Nei, 1987) of 1000 sampled datasets. Only bootstrap values of more than 50% are shown. GenBank sequence accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

41451<sup>T</sup> and 95.9% similarity (56/1354) to *S. macrosporus* DSM 41449<sup>T</sup>. The position of strain VC-A46<sup>T</sup> in the phylogenetic tree was unaffected by the choice of tree-making algorithm and the strains used as the outgroup. These results suggest that strain VC-A46<sup>T</sup> represents a novel species that is closely related to the above three *Streptomyces* species.

The variable  $\gamma$ -region sequences (158–277 nt) of the 16S rRNA gene from strain VC-A46<sup>T</sup> and 245 *Streptomyces* species (only type strains were used) in GenBank were aligned (Supplementary Fig. S2 in IJSEM Online). Analysis of  $\gamma$ -region sequences indicated that the strain VC-A46<sup>T</sup> was clustered with *S. thermolineatus* DSM 41451<sup>T</sup>, *S. macrosporus* DSM 41449<sup>T</sup> and *S. megasporus* DSM 41476<sup>T</sup>; sequence similarity values were respectively 94.4, 91.2 and 89.6%. Strain VC-A46<sup>T</sup> also had some phenotypic differences from these recognized *Streptomyces* species in the same branch. Thus, phylogenetic study supports the placement of strain VC-A46<sup>T</sup> in a novel species.

Levels of DNA–DNA relatedness between strain VC-A46<sup>T</sup> and four closely related species (based on the above phylogenetic data), *S. thermolineatus* DSM 41451<sup>T</sup>, ‘*S. cattleya*’ JCM 4925, *S. macrosporus* DSM 41449<sup>T</sup> and *S. acidiscabies* ATCC 49003<sup>T</sup>, were 21.5, 39.8, 19.8 and 60.6%, respectively (Supplementary Table S2 in IJSEM Online). DNA–DNA relatedness values below 80% have been recommended for the recognition of novel genomic species of *Streptomyces* (Labeda, 1993, 1996, 1998). The observed levels of DNA–DNA relatedness values demonstrate the genomic distinction of strain VC-A46<sup>T</sup> from *S. thermolineatus*, ‘*S. cattleya*’, *S. macrosporus* and *S. acidiscabies*.

### Description of *Streptomyces cheonanensis* sp. nov.

*Streptomyces cheonanensis* (che.on.an.en’ sis N.L. masc. adj. *cheonanensis* pertaining to Cheonan, Republic of Korea, the geographical origin of the type strain).

Aerobic, Gram-positive, non-motile actinomycete that forms extensively branched aerial and substrate hyphae. Short or long, straight to flexuous chains of smooth-surfaced spores are evident on the aerial hyphae. The aerial mycelium is grey to white in colour and the substrate mycelium appears light yellow when grown on ISP 4 agar. The cell wall contains LL-diaminopimelic acid. Predominant cellular fatty acids are 14-methylpentadecanoic acid (iso-C<sub>16:0</sub>; 47.82%), hexadecanoic acid (C<sub>16:0</sub>; 14.44%) and *cis*-9-hexadecenoic acid (C<sub>16:1 cis</sub> 9; 10.24%). The G+C content of the genomic DNA is 75.5 mol%. Optimum growth occurs at 29 °C. Grows well in yeast extract/malt extract broth adjusted to pH 6.5–8.0. Tolerates NaCl concentrations up to 7%. Aerial and substrate mycelia grow abundantly on both ISP 3 agar and Bennett’s agar. Soluble pigments are generated on ISP 2, ISP 3, ISP 5 (glycerol/asparagine agar) and ISP 7. Capable of utilizing several carbon sources, including adonitol, arabinose, dextran, fructose, *myo*-inositol, mannitol, D-melezitose, D-melibiose, raffinose, L-rhamnose, sucrose, xylitol and xylose. Can also use several nitrogen sources: DL- $\alpha$ -amino-n-butyric acid, L-cysteine, L-histidine, L-hydroxyproline, L-phenylalanine and L-valine. Resistant to penicillin G, but sensitive to neomycin, oleandomycin and rifampicin. Secretes compounds that inhibit mycelial growth of plant-pathogenic

fungi including *Alternaria mali*, *Colletotrichum orbiculare*, *Magnaporthe grisea*, *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* and the oomycete *Phytophthora capsici*.

The type strain is strain VC-A46<sup>T</sup> (=KCCM 42119<sup>T</sup>=NBRC 100940<sup>T</sup>), isolated from a soil sample collected from Cheonan, Korea.

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