***Kitasatospora cheerisanensis* sp. nov., a new species of the genus *Kitasatospora* that produces an antifungal agent**

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An actinomycete, strain YC75^T, which produced bafilomycin-like antifungal compounds, was identified as a member of the genus *Kitasatospora* on the basis of morphological and chemotaxonomic characteristics. The strain produced the aerial and fragmenting vegetative mycelia consisting of straight chains of 20 or more smooth-surfaced spores. Submerged spores were formed in tryptic soy broth. No soluble pigments were formed. Whole-cell hydrolysates contained glucose and mannose, but not galactose. The 16S rDNA sequence of YC75^T was compared with those of the other representative *kitasatosporae* and streptomycetes. Strain YC75^T formed a significant monophyletic clade with *Kitasatospora phosalacinea*. The levels of DNA relatedness between strain YC75^T and representatives of the genus *Kitasatospora* ranged from 16 to 59% including *K. phosalacinea* (28 and 40%). It is clear from polyphasic evidence that the isolate should be classified as *Kitasatospora cheerisanensis* sp. nov., whose type strain is YC75^T (= KCTC 2395^T). The presence of galactose in whole-cell hydrolysates may not be a stable chemical marker for the genus *Kitasatospora*.

Keywords: *Kitasatospora cheerisanensis* sp. nov., antifungal agent, bafilomycin, actinomycete

INTRODUCTION

The genus *Kitasatospora* comprises a group of filamentous and aerobic Gram-positive bacteria with the aerial mycelium bearing long spore chains of more than 20 spores and is phenotypically similar to *Streptomyces* strains (Omura *et al.*, 1982). Differential characteristics of the genus *Kitasatospora* which can be used to distinguish it from the genus *Streptomyces* are the higher ratio of *meso*-diaminopimelic acid (DAP) to LL-DAP and the presence of galactose in the whole-cell hydrolysates (Omura *et al.*, 1982; Zhang *et al.*, 1997). Wellington *et al.* (1992) proposed to unify two taxa on the basis of rRNA-targeted oligonucleotide probes and phenotypic properties. This proposal was later supported by ribosomal protein AT-L30 analysis (Ochi

& Hiranuma, 1994). In a recent phylogenetic study based on complete 16S rDNA sequence data, Zhang *et al.* (1997), however, proposed to revive the genus *Kitasatospora* as they form a significant monophyletic clade and contain differential chemotaxonomic markers, notably relatively larger amount of *meso*-DAP and galactose in their cell walls. *Kitasatospora* species, like streptomycetes, have been known to produce several antifungal and antibacterial agents (Kusakabe & Kiyoshi, 1988; Nakamura *et al.*, 1989). Several antifungal agents, such as validamycin and polyoxin produced by *Streptomyces* species, were developed and now are commercially available for agricultural fungicides (Yamaguchi, 1995).

An actinomycete strain, designated YC75, was isolated from a soil humus sample collected from Cheerisan, a mountain in the Republic of Korea, in the process of screening biological control agents for the plant-pathogenic fungus *Rhizoctonia solani* (Sung & Chung, 1997). The strain produced an antifungal agent which inhibits mycelial growth of several important plant

Abbreviations: DAP, diaminopimelic acid; ISP, International *Streptomyces* Project.

The GenBank accession number for the 16S rDNA sequence of strain YC75^T is AF050493.

pathogens, namely *Botrytis cinerea*, *Colletotrichum lagenarium*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. The taxonomic status of strain YC75^T was investigated using a combination of phenotypic, chemical and molecular systematic means. On the basis of polyphasic evidence, we propose strain YC75^T be classified as *Kitasatospora cheerisanensis* sp. nov.

METHODS

Bacterial strain. Strain YC75^T was isolated from a soil humus sample collected at Cheerisan mountain, Republic of Korea, using standard dilution agar plate methods. Aliquots of serially diluted soil suspension were inoculated onto trypticase soy agar (TSA; BBL) plates, followed by incubation at 28 °C for 3 d. Bacterial colonies were picked up and their inhibitory activities against *R. solani* were determined by paired bioassay on TSA plates. An isolate, designated YC75^T, showed substantial inhibition on mycelial growth of the fungus and was studied further. The reference strains were obtained from Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, Taejeon, Korea. The test strains were maintained on YM agar (l⁻¹: 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose, 20 g agar) or Bennett's agar (l⁻¹: 1 g yeast extract, 1 g beef extract, 2 g N-Z amine type A, 10 g glucose, 20 g agar).

Cultural and morphological characteristics. Cultural properties were examined by 14-d-old cultures grown on the media recommended by the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966). Morphological characteristics were examined using light microscopy and scanning electron microscopy (JEOL model JSM-6400).

Chemotaxonomy. Analyses of diaminopimelic acid and sugars were carried out according to Lechevalier & Lechevalier (1970). The biomass for cellular fatty acid analysis was prepared from one d-old culture grown on TSA plate at 28 °C. Fatty acid methyl esters were prepared using the method described in the manual of Hewlett Packard Microbial Identification System (MIDI). The resultant esters were separated using a gas chromatograph (model 5890; Hewlett Packard) fitted with a methyl phenyl silicon fused silica capillary column (25 m by 0.2 mm; Hewlett Packard). Phospholipids were extracted from freeze-dried cells with chloroform/methanol/0.3% aqueous sodium chloride (9:10:3, by vol.) and detected by two-dimensional TLC according to Embley & Wait (1994). Isoprenoid quinones were analysed by using mass spectrometry (Collins, 1994).

Physiological tests. Catalase activity, acid production from carbohydrates, utilization of carbohydrates as the sole carbon source for growth, hydrolysis and decomposition of casein, cellulose, starch, chitin, aesculin, gelatin, hippurate, hypoxanthine, tyrosine and Tween 40 were performed by the methods described by Smibert & Krieg (1981). Growth in the presence of sodium chloride (1, 3 and 5%, w/v) and sensitivity to antibiotics were determined on nutrient agar at 28 °C. The temperature and pH ranges for growth were tested on nutrient agar.

DNA–DNA hybridization. Chromosomal DNA was prepared following the method of Marmur (1961). The levels of DNA relatedness between test strains were measured using the procedure of Sambrook *et al.* (1989). Duplicate aliquots containing 200 ng of each denatured genomic DNA were

transferred onto positively charged nylon membranes (Hybond-N+; Amersham) by using dot blot apparatus (Bio-Rad). The membranes were air-dried and cross-linked in a UV illuminator for 1 min. Twenty-five nanograms of DNA were denatured by boiling for 5 min, labelled using *rediprime* DNA-labelling system (Amersham) with Redivue [³²P]dCTP (Amersham), and purified by using NucTrap Probe Purification Columns (Stratagene). The membranes were prehybridized in hybridization solution (5× Denhardt's reagent, 6× SSC, 0.5% SDS, 100 µg denatured fragmented salmon sperm DNA ml⁻¹, and 35% formamide) at 65 °C for 1 h. The labelled probe DNAs were then added to the hybridization solutions and incubated at 65 °C for 18 h. The resultant membranes were washed at 65 °C for 5 min with 2× SSC and 0.1% SDS. After 24 h exposure of the membrane at -70 °C, the signal intensities were measured using a densitometer (Bio-Rad) and peak-areas determined by 1-D Analyst II software (Bio-Rad). The signal produced by self-hybridization of the probe with homologous target DNA was taken as 100% and the percentage homology values calculated for the duplicated slots.

DNA base composition. The G + C content of the DNA was determined from the midpoint value of the thermal denaturation profile using a spectrophotometer (Ultrospec 2000; Pharmacia Biotech) equipped with a programmable peltier temperature-control unit by the equation of Marmur & Doty (1962), as modified by De Ley (1970).

16S rDNA sequencing. The 16S rDNA was enzymically amplified using two oligonucleotide primers based on published sequences (Collins *et al.*, 1994). The sequences of the two primers are 5' TATGGATCCTTCTACGGAGAGTTTGATCC 3' and 5' TATGGATCCCCACCTTCCGGTACGGCTACC 3' (*Bam*HI sites are in bold). The temperatures for denaturation, annealing and extension were 92, 55 and 72 °C, respectively. The PCR products were digested with *Bam*HI and cloned into the same site of the pBluescript II SK(+) vector. Overlapping subclones were generated by exonuclease III digestion of the insert DNA. The nucleotide sequences of these clones were determined using the Sequenase version 2.0 kit (USB). The resultant 16S rDNA sequence of strain YC75^T was manually aligned with representative sequences (positions 32–71, 98–1135, 1140–1450 and 1454–1507 of *E. coli* numbering) of actinomycetes obtained from the Ribosomal Database Project (Maidak *et al.*, 1997) and GenBank databases. Phylogenetic trees were inferred by using four treeing algorithms, namely the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant unrooted tree topology was evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

RESULTS

Morphological characteristics

Strain YC75^T produced the aerial mycelium which consisted of straight chains of 20 or more rod-shaped smooth-surfaced spores (0.75–0.90 by 1.2–1.5 µm)

Fig. 1. Scanning electron micrograph showing the smooth surfaces of spores of strain YC75^T (= KCTC 2395^T). The organism was grown on potato dextrose agar at 28 °C for 14 d. Bar, 1 µm.

(Fig. 1). The vegetative mycelia grown in TSA or trypticase soy broth (TSB) were easily fragmented and submerged spores were formed in TSB. The isolate grew well on various organic media and produced abundant aerial spores on inorganic salt-starch agar (ISP medium 4), tyrosine agar (ISP medium 7), potato-dextrose agar and Bennett's agar plates (Table 1). The spore mass was whitish-grey and the reverse sides of colonies were greenish-yellow on most agar media. No soluble pigments were produced. Colonies lacking aerial mycelium during early growth stage were pasty, circular, convex and dull milky on 0.1 × TSA plate.

Chemotaxonomy

Strain YC75^T contained *meso*-DAP and *LL*-DAP in the whole-cell hydrolysate. No galactose was detected, but glucose and mannose were present in the whole-cell hydrolysate. Phosphatidylethanolamine, phosphatidylinositol, diphosphatidyl glycerol and six

unidentified phospholipids were contained in the polar lipid fraction. The predominant fatty acids in whole-cell methanolysates were 15:0 *iso* (35.4%), 15:0 *anteiso* (18.3%), 16:0 (10.9%) and 17:0 *iso* (8.3%). The major menaquinone type was MK-9(H₈). The G + C content of the DNA was 75.8 mol %.

Physiological characteristics

Following characteristics of strain YC75^T were positive: catalase activity, reduction of nitrate, production of acid from arabinose, cellobiose, galactose, glucose, maltose, trehalose and xylose. Casein, chitin, chitosan, starch, aesculin, gelatin, hippurate and Tweens 40, 60 and 80 were decomposed, but cellulose, hypoxanthine and tyrosine were not decomposed. Growth occurred in the presence of 1 %, but not in 3 % sodium chloride. The growth also occurred at pH 4–9 and the temperature range of 20–37 °C. Strain YC75^T utilized arabinose, azelate, cellobiose, cronate, fumarate, D-glucose, galactose, *iso*-butyrate, malonate, maltose, rhamnose, trehalose and xylose for growth, but not adonitol, fructose, glycerol, mannitol, *myo*-inositol, melezitose, raffinose, sorbitol, suberate, succinate or sucrose. This strain was susceptible to ampicillin, chloramphenicol, kanamycin, nalidixic acid, oxytetracycline, rifampicin and streptomycin.

The strain YC75^T inhibited mycelial growth of several plant-pathogenic fungi, such as *R. solani*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Pythium ultimum*, *Colletotrichum lagenarium*, *Magnaporthe grisea* and *Botryosphaeria dothidea* by producing bafilomycin-C1-like antifungal compounds (personal communications with Dr S. S. Moon, Kongju National University, Korea).

Molecular systematics

An almost complete 16S rDNA sequence was determined for strain YC75^T (1514 nucleotides). The preliminary phylogenetic analysis based on a large

Table 1. Morphological characteristics of strain YC75^T

All characteristics were observed after 2 weeks incubation at 28 °C. On all media: growth was good; where present, spore mass colour was whitish-grey; and soluble pigment was absent.

Medium	Sporulation	Reverse colour
Yeast extract/malt extract agar (ISP No. 2)	Moderate	Greenish-yellow
Oatmeal agar (ISP no. 3)	Moderate	Greyish-white
Inorganic salt-starch agar (ISP No. 4)	Abundant	Greyish-white
Glycerol-asparagine agar (ISP No. 5)	Poor	Grey
Peptone/yeast extract iron agar (ISP No. 6)	None	Greenish-yellow
Tyrosine agar (ISP no. 7)	Abundant	Greenish-yellow
Potato dextrose agar	Abundant	Deep yellow
Tryptic soy agar	None	Greenish-yellow
Nutrient agar	None	Greenish-yellow
Bennett's agar	Abundant	Greenish-yellow
Starch agar	Moderate	Greenish-yellow

Fig. 2. Unrooted neighbour-joining tree based on 1445 unambiguously aligned nucleotide positions including positions 32–71, 98–1135, 1140–1450 and 1454–1507 of *E. coli* numbering. Asterisks indicate the branches that were also recovered in the remaining three methods employed in this study (see text for details). The numbers at the nodes exhibit the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets. The corresponding accession numbers are given in parentheses. Bar represents 0.01 nucleotide substitution per position.

Table 2. DNA relatedness of strain YC75^T to representatives of the genus *Kitasatospora* species

Strain	Labelled DNAs			
	YC75 ^T	KCTC 9746 ^T	KCTC 9745 ^T	KCTC 9735 ^T
Strain YC75 ^T	100	26	46	28
<i>K. cystarginea</i> KCTC 9746 ^T	16	100	12	19
<i>K. griseola</i> KCTC 9745 ^T	59	36	100	28
<i>K. mediocidica</i> KCTC 9733 ^T	18	19	23	9
<i>K. phosalacinea</i> KCTC 9735 ^T	40	33	61	100
<i>K. setae</i> KCTC 9182 ^T	42	35	59	42

data set consistently placed strain YC75^T in a clade corresponding to the genus *Kitasatospora* (data not shown). When the phylogenetic position of isolate YC75^T was investigated with all of validly described species of the genus *Kitasatospora* and representatives of the genus *Streptomyces*, the organism formed a monophyletic clade with the type strain of *Kitasatospora phosalacinea* (Fig. 2). The relationship was evident in evolutionary trees based on four different treeing methods and also supported by a high bootstrap value of 84%. Nucleotide sequence similarity values between the isolate and kitasatosporae range from 97.0% (*Kitasatospora azatica*) to 98.5% (*K. phosalacinea*). *Kitasatospora phosalacinea* and *Kitasatospora griseola* (98.1%) were the only species that represent more than 98% sequence similarity. The genealogical position of isolate YC75^T was further examined using DNA–DNA pairing study. DNA relatedness values between our isolate and representatives of the genus *Kitasatospora* ranged from 16% (with *Kitasatospora cystarginea*) to 59% (*K. griseola*) (Table 2).

DISCUSSION

It is clear from phylogenetic analyses based on 16S rDNA sequences that strain YC75^T belongs to the genus *Kitasatospora*. In a comparison of some phenotypic characteristics of strain YC75^T with those of phylogenetically related *Kitasatospora* species,

Kitasatospora azatica, *Kitasatospora cochleata*, *K. cystarginea*, *K. griseola*, *Kitasatospora mediocidica*, *Kitasatospora setae*, *Kitasatospora paracochleata* and *K. phosalacinea*, strain YC75^T had unique differential properties as shown in Table 3 (Labeda, 1988; Nakagaito *et al.*, 1992; Nakamura *et al.*, 1989). Only strain YC75^T liquified gelatin and produced bafilomycin-C1-like antifungal agents. Many *Kitasatospora* species were known to produce anti-fungal and antibacterial agents such as cystargin and setamycin (Kusakabe & Kiyoshi, 1988; Omura *et al.*, 1989). Rhamnose is utilized only by strain YC75^T and *K. phosalacinea*, which is most closely related to the strain YC75^T in the phylogenetic analysis. The presence of galactose in the whole-cell hydrolysate, which distinguishes the genus *Kitasatospora* from the genus *Streptomyces*, was not confirmed even by the analysis using HPLC in our study (Omura *et al.*, 1982; Zhang *et al.*, 1997). Instead the strain YC75^T was found to contain glucose and mannose in the whole-cell hydrolysate. This suggests that the presence of galactose may not be a good criterion for recognizing the genus *Kitasatospora*, as our isolate was clearly assigned to the genus based on 16S rDNA analysis. As shown in Fig. 2, 16S rDNA analysis showed not only that strain YC75^T is an authentic member of the genus *Kitasatospora* but also that the closest phylogenetic neighbour is *K. phosalacinea*. DNA relatedness values lower than 59% with five *Kitasatospora* species including *K. phosalacinea* further indicate that the organism is

Table 3. Morphological, physiological and biochemical characteristics of strain YC75^T and phylogenetically related *Kitasatospora* species

+, Positive; —, negative; v, variable.

Character	Strain YC75 ^T	<i>K. azatica</i> *	<i>K. cochleata</i> *	<i>K. cystarginea</i> *	<i>K. griseola</i> †	<i>K. mediocidica</i> ‡	<i>K. setae</i> *	<i>K. paracochleata</i> *	<i>K. phosalacinea</i> *
Aerial mycelia	<i>Rectiflexibiles</i>	<i>Rectiflexibiles</i>	Hook to spirales	<i>Spirales</i>		<i>Rectiflexibiles</i>	<i>Rectiflexibiles</i>	<i>Spirales</i>	<i>Rectiflexibiles</i>
Soluble pigment	—	—	Yellowish-brown	—	Pink	Brown	—	Yellowish-brown	—
Melanin formation	—	—	+	—	—	—	—	+	—
Nitrate reduction	+	+	—	—	—	—	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	—	—	—	—	—	—	—	—
Antibiotic produced†	Bafilomycin	Azaamino acid		Cystargin	Setamycin	Mediocidin	Setamycin		Phosalacine
Utilization of carbohydrates:									
L-Arabinose	+	+	+	—	+	+	+	—	—
D-Fructose	—	+	—	—	—	+	—	+	+
D-Glucose	+	+	+	+	+	+	+	+	+
Inositol	—	—	—	—	—	—	—	—	—
D-Mannitol	—	—	—	—	—	v	—	—	—
Raffinose	—	—	—	—	+	—	—	—	—
L-Rhamnose	+	—	—	—	—	—	—	—	+
Sucrose	—	—	—	—	—	+	—	—	—
D-Xylose	+	+	—	—	+	+	+	—	+
Galactose in whole cells	—	+	+	+	+	+	+	+	+
G + C content (mol %)	75.8	70.5	72.4	70.6	66.0	74.0	73.2	73.1	73.3

* Data from Nakagaito *et al.* (1992).† Data from Nakamura *et al.* (1989).

‡ Data from Labeda (1988).

genetically distinctive and forms a genomic species in the genus *Kitasatospora*, which satisfies the critical value proposed by Wayne *et al.* (1987). We therefore propose the new name *Kitasatospora cheerisanensis* for strain YC75^T on the basis of the phenotypic, chemical and molecular systematic data.

Description of *Kitasatospora cheerisanensis* sp. nov.

Kitasatospora cheerisanensis (chee.ri.sanen'sis. M.L. masc. adj. *cheerisanensis* pertaining to Cheerisan, the name of a mountain in Korea where the species was originated).

Aerobic, Gram-positive, non-acid-fast actinomycete which produces a greenish-yellow substrate mycelium and a whitish-grey aerial mycelium on yeast extract-malt extract agar, inorganic salt-starch agar, tyrosine agar, Bennett's agar and starch agar. Vegetative mycelia fragment into bacillary structures. Spore chains are *rectiflexibiles*, with 20 or more rod-shaped smooth-surfaced spores (0.75–0.90 by 1.2–1.5 µm) per chain. Submerged spores are formed in liquid culture. Soluble pigments including melanin are not produced. Colonies lacking aerial mycelium during early growth stage were pasty, circular, convex and dull milky on 0.1 × TSA. The cell wall contains *meso*-DAP and *LL*-DAP. Whole-cell hydrolysates contain glucose and mannose, but lack galactose. Phosphatidylethanolamine, phosphatidylinositol, diphosphatidylglycerol are contained in the polar lipid fraction. The predominant fatty acids are saturated iso- and anteiso-branched as well as straight-chain fatty acids. The major menaquinone type is MK-9(H₈). The strain is positive for catalase activity and reduction of nitrate.

Acids are produced from arabinose, cellobiose, galactose, glucose, maltose, trehalose and xylose. Casein, chitin, chitosan, starch, aesculin, gelatin, hippurate and Tweens 40, 60 and 80 are decomposed, but cellulose, hypoxanthine and tyrosine are not. Growth occurs in the presence of 1% NaCl, but not in 3% NaCl. Good growth occurs at pH 7–8, and the optimum temperature range is 25–30 °C. Arabinose, azelate, cellobiose, cronate, fumarate, D-glucose, galactose, iso-butyrate, malonate, maltose, rhamnose, trehalose and xylose are utilized for growth, but adonitol, fructose, glycerol, mannitol, *myo*-inositol, melezitose, raffinose, sorbitol, suberate, succinate and sucrose are not utilized. This strain is susceptible to ampicillin, chloramphenicol, kanamycin, nalidixic acid, oxytetracycline, rifampicin and streptomycin. The type strain YC75^T produced bafilomycin-C1-like antifungal compounds. The G + C content of the DNA is 75.8 mol%. The type strain is *Kitasatospora cheerisanensis* YC75^T and has been deposited in Korean Collection for Type Cultures as KCTC 2395^T.

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