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Tsukamurella sunchonensis sp. nov., a Bacterium Associated with Foam in Activated Sludge

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The taxonomic position of actinomycete strain SCNU5^T, isolated from extensive foam in the aeration basin of an activated sludge process, was clarified by phenotypic, chemotaxonomic and phylogenetic analyses. The strain possesses wall chemotype IV, MK-9(H_0), as the major menaquinone, and contains saturated, monounsaturated and 10-methyl branched fatty acids. The G+C content of its DNA is 68.1 mol%. Phenotypic data and DNA relatedness to known species indicate that the strain SCNU5^T represents a new species within the genus *Tsukamurella*, for which we propose the name *Tsukamurella sunchonensis* sp. nov. The type strain of *T. sunchonensis* is SCNU5^T (=KCTC 9827^T).

Key words: *Tsukamurella sunchonensis* sp. nov., foam, activated sludge

The pathogenic and taxonomic history of the genus *Tsukamurella*, particularly that of *Tsukamurella paurometabola, T. inchonensis, T. pulmonis, T. tyrosinosolvens,* and *T. strandjordae* have been outlined in detail (Yassin *et al.*, 1995; Yassin *et al.*, 1996; Yassin *et al.*, 1997; Kattar *et al.*, 2001). *T. paurometabola* has been isolated from the sputum of a patient with a tuberculosis-like disease (Tsukamura and Kawakami, 1982; Tsukamura *et al.*, 1988), and from fetal meningitis, severe gangrenous tendosynovitis and bacteremia. *T. inchonensis* was isolated from blood cultures of a patient who had ingested hydrochloric acid and from the blood culture of lung carcinoma (Chong *et al.*, 1997; Yassin *et al.*, 1995).

T. pulmonis was isolated from sputum of a patient with pulmonary tuberculosis (Yassin *et al.*, 1996), and *T. tyrosinosolvens* was isolated from a blood culture of a patient with cardiac pacemaker implants (Yassin *et al.*, 1997). Finally, *T. strandjordae* has been isolated from blood cultures of acute myelogenous leukemia (Kattar *et al.*, 2001).

Chemotaxonomic data indicate that the genus is closely related to the mycolic acid containing actinomycete genera, such as, *Mycobacterium, Nocardia, Corynebacterium, Dietzia, Rhodococcus, and Gordona.* All of these taxa have a cell wall chemotype (Lechevalier and Lechevalier, 1970), a fatty acid profile containing as major components, straight-chain saturated, unsaturated and tuberculostearic acids, and contain menaquinones as the only respiratory isoprenoid quinones. However, differences in mycolic acid sizes and menaquinone compositions are very valuable for differentiating between *Tsukamurella* and related taxa (Yassin *et al.*, 1996; Yassin *et al.*, 1997). Phylogenetically, the genus *Tsukamurella* is related to other mycolic acid-containing actinomycetes.

The present investigation was designed to establish the taxonomic position of an isolate, designated SCNU5^T, which is associated with extensive foaming in aeration basins. The chemical, morphological and physiological properties of the strain were determined. In addition, the almost complete sequences of the 16S rRNA gene (rDNA) of the isolate were determined and compared with related species using a tree-making algorithm (Saitou and Nei, 1987). The DNA relatedness of the strain with type strains was also determined. Polyphasic taxonomic data indicate that strain SCNU5^T (KCTC9827^T) is a new species of *Tsukamurella,* for which we propose the name *Tsukamurella sunchonensis* sp. nov.

Materials and Methods

Bacterial strains

The strain SCNU5^T was isolated from foam collected from a full-scale activated sludge plant in Sunchon, Korea (Seong *et al.*, 1999). *Tsukamurella pulmonis* DSM 44142^T,

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Tsukamurella tyrosinosolvens DSM 44234^T, *and Tsukamurella inchonensis* DSM 44067^T, were obtained from the German Collection of Microorganisms and Cell Cultures.

Morphology and physiological characteristics

Strain SCNU5^T was grown on glucose yeast extract agar (GYEA), yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), and inorganic salts-starch casein agar (ISP medium 4) (Shirling and Gottlieb, 1966). Cultures were conducted at 30°C for 7 days, and were examined daily for aerial mycelium color, pigmentation and colony morphology. Melanin production was observed on peptone-yeast extract-iron agar (ISP6) and tyrosine agar (ISP7). An air-dried smear from brain heart infusion (BHI) agar culture was stained using the Gram and Zieh Neelsen methods in order to determine the Gram reaction and acid fastness, respectively. morphological observations of cells were made with a light microscope and a model JSM5310 electron microscope (JOEL).

The strain was examined for 95 unit characters, including; pH and temperature-dependent growth, enzyme activity, resistance to antibiotics and chemicals, antibiosis, utilization of carbon sources and acid production from sugars (Gordon *et al.*, 1974; Williams *et al.*, 1983; Yassin *et al.*, 1996; Yassin *et al.*, 1997).

Chemotaxonomy

Biomass was obtained from liquid culture in Sauton's broth medium (Difco) at 28°C for 5 days, freeze dried and kept refrigerated for further analysis. To determine the acyl types on the muramyl residues of peptidoglycan, glycolate extraction and detection were performed, as described previously (Uchida and Seino, 1997; Uchida *et al.*, 1999).

Mycolic acids were extracted by alkaline methanolysis (Minnikin *et al.*, 1980). Thin layer chromatography (Kieselgel 60 aluminium plate (Merck)) was used to detect the mycolic acids, using a single development with petroleum ether (bp 60°C-80°C)-acetone (95:5, v/v), and double development with toluene-acetone (97:3, v/v). Detection was performed with a 5% ethanolic molybdophosphoric acid spray followed by heating at 180°C for 5 min.

For the extraction of menaquinone, the biomass was treated as described in Kim *et al.* (1996). High performance liquid chromatographic separation of quinones was performed using an ODS Hypersil column (200×4.6 mm, particle size 5 μm, Hewlett Packard) with acetonitrile/tetrahydrofuran (70:30, v/v) as the mobile phase. The flow rate was 1 ml/min at 37°C, and UV detector was operated at 254 nm.

Whole cell sugars were extracted as alditol acetates (Englyst and Cummings, 1984), and analyzed by gas chromatography (GC) (Hewlett Packard 5890A) fitted with a flame ionization detector (FID), and a 0.53 mm× 30 m SP 2380 (Supelco) fused silica capillary column using the following temperature program; constant 160°C for 2 min followed by a programmed rise of 5°C/min. The injector temperature was held at 250°C, and the detector at 300°C.

Fatty acids as their methyl esters were extracted by alkaline methanolysis (Gordon *et al.*, 1974). Fatty acid methyl esters (FAMEs) were separated using a HP-1 capillary column (0.53 mm I.D., 30 m length, 2.65 μm film) in the HP 5809A GC equipped with an FID. The temperature program was constant at 170°C for 1 min, followed by a programmed rise of 5°C/min. The injector temperature was held at 250°C, and the detector at 300°C.

Molecular systematics

Chromosomal DNA was isolated from the test strains using a procedure (Chun, 1995), which is a slight modification of that described in Pitcher *et al.* (1989). The G+C content of the DNA preparation was determined using the thermal denaturation (*Tm*) method (Chun and Goodfellow, 1995). DNA-DNA hybridization was carried out using the slot-dot blot method (Kafatos *et al.*, 1979), and a DIG High Prime DNA Labeling and Detection Starter Kit (Boehringer Manheim, Germany) was used for labeling the genomic DNA and for signal detection. Data were analyzed using the TINA 2.0 program.

PCR amplification of 16S rDNA was performed using a capillary PCR machine (Rapidcycler; Idaho Technology, USA), and the previously described 27f and 1525r primers (Chun, 1995), as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1min and elongation at 72°C for 3 min for each cycle. A total of 30 cycles were performed and these were followed by a final elongation step at 72°C for 10 min and a cooling at 25°C for 1 min. The amplified fragments were directly sequenced using a Taq DyeDeoxy terminator Cycle Sequencing Kit (Applied Biosystems, USA), and the previously described oligonucleotide primers (Chun, 1995). Sequencing gel electrophoresis was carried out and the nucleotide sequences were obtained automatically using an Applied Biosystems DNA sequencer (model 373A) and the software provided by the manufacturer.

The 16S rDNA sequences were aligned manually with mycolic acid containing actinomycete nucleotide sequences derived from the Ribosomal Database Project (Maidak *et al.*, 1996) and EMBL/GenBank database using the AL 16S program (Chun, 1995). The reference sequences had the following accession numbers: Z46753 (*Corynebacterium glutamicum*), X79290 (*Dietzia maris*), X80633 (*Gordonia aichiensis*), X80635 (*G. amarae*), X79287 (*G. bronchialis*), X80632 (*G. rubropertincta*), X80634 (*G. sputi*), X79286 (*G. terrae*), X52917 (*Mycobacterium tuberculosis*), X57949 (*Nocardia asteroides*), X79288 (*Rhodococcus rhodochrous*), Z46751 (*Tsukamurella paurometabola*), X87340 (*G. hydrophobica*), X93485 (*G. hirsuta*), Z35435 (*Skermania piniformis*),

X92981 (*T. pulmonis*), and Y12246 (*T. tyrosinosolvens*), AF283283 (*T. strandjordae*), and X85955 (*T. inchonensis*). 16S rDNA sequence of strain SCNU5^T was deposited in the GenBank under accession number AF150494. The evolutionary tree for the datasets was inferred using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distance matrix for the neighbor-joining method was generated as described by Jukes and Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for constructing the tree.

Results and Discussion

Morphological characteristics of the isolate

Strain SCNU5^T grew well on ISP medium 2; the colonies were orange colored and rough. On ISP medium 3 and ISP medium 4, growth was weak and leathery. No diffusible pigments were observed. Growth occurred at 20°C to 30°C within 3 to 4 days on BHI agar and on GYE agar. Dilute inocula on BHI agar yielded large, eugonic, cream-colored, rough colonies. Spores, capsules, true branching and aerial hyphae were not observed. Cells of strain SCNU5^T were long rod shaped, Gram-positive and slightly acid-alcohol fast. During growth, the cells appeared primarily as rods, which stuck together, either pole to pole or pole to side, to produce a branched hyphae like arrangement. These rods appeared to fragment by septation. The colonial and cellular morphology of this strain were typical of strains belonging to the genus *Tsukamurella* (Yassin *et al.*, 1996; Yassin *et al.*, 1997).

Physiological characteristics

The physiological properties of strain SCNU5^T are described in Table 1. This strain did not produce aerial mycelium, diffusible pigment or melanin. It grew at pH 9, but not at pH 4. Its growth temperature range was 28°C~37°C, and it did not grow at 10°C. SCNU5^T did not grow on MacConkey agar, but grew in the presence of 7% NaCl and 0.1% phenol. SCNU5^T degraded esculin, hypoxanthine and tyrosine, but not casein, gelatin, starch or urea. The strain had a catalase, but no lipase, pectinase or nitrate reductase. SCNU5^T was sensitive to streptomycin, gentamicin, and vancomycin, but resistant to bacitracin and penicillin G. SCNU5^T showed no antimicrobial activity, and utilized sucrose, meso-inositol, mannitol, D-melezitose, D-galactose, citrate, succinate, pyruvate and propionate, but not benzoate, lactate or oxalate. SCNU5^T produced acids from glucose, trehalose, xylose, meletzitose, sucrose, fructose and maltose, but not from maltose, raffinose, adonitol or mannose.

Chemotaxonomy

SCNU5^T contained meso-diaminopimelic acid (*meso*-DAP). Two-dimensitonal thin-layer chromatography of whole-cell alkaline methanolysates of strain SCNU5^T

Table 1. Diagnostic physiological characteristics of isolate SCNU5^T, *T. tyrosinosolvens* DSM 44234 ^{T} and *T. pulmonis* DSM 44142^T

Unit Characters	Strain SCNU5^T	*T. tyrosinosolvens* DSM 44234^T	*T. pulmonis* DSM 44142^T
Growth at			
4°C	–	–	–
10°C	–	+	+
28°C	+	+	+
37°C	+	+	+
45°C	–	–	–
pH 4	–	–	–
pH 7	+	+	+
pH 9	+	+	+
MacConkey ager	–	+	+
Resistance to antibiotics (μg/ml)			
Streptomycin (10)	–	ND[a]	ND
Bactracin (10 i.u.)	+	+	+
Gentamicin (10)	–	–	–
Tobramycin (10)	–	+	+
Penicillin G (10 i.u.)	+	+	+
Vancomycin (30)	–	–	–
Rifampicin (50)	–	–	–
Utilization as sole carbon sources			
Benzoate	–	+	–
Lactate	–	–	–
Citrate	–	+	–
Sucrose	+	+	+
Meso-inositol	+	+	–
Mannitol	+	+	–
Rhamnose	–	–	–
Raffinose	–	–	–
Melezitose	+	+	–
Adonitol	–	–	–
Melibiose	–	–	–
Dextran	–	–	–
Arabinose	–	–	–
Xylose	–	–	–
Fructose	+	+	+
Lactose	–	–	–
Galactose	+	+	+
Hippurate	–	–	–
Erythritol	–	–	–
Glucose	+	+	+
Trehalose	+	+	+
Adonitol	–	–	–
Arabinose	–	–	–
Ducilitol	–	+	–
Maltose	+	+	–

Table 1. Continued

Unit Characters	Strain SCNU5^T	*T. tyrosinosolvens* DSM 44234^T	*T. pulmonis* DSM 44142^T
Acid production from carbohydrates			
Glucose	+	+	+
Trehalose	+	+	+
Raffinose	–	–	–
Xylose	–	–	–
Adonitol	–	–	–
Sorbitol	–	+	+
Melezitose	+	+	–
Melibiose	–	–	–
Arabinose	–	–	–
Sucrose	+	+	+
Mannose	–	+	+
Fructose	+	+	+
Dextran	–	–	–
Erythritol	–	–	–
Lactose	–	–	–
Xylitol	–	+	–
Maltose	+	+	–
Antimicrobial activity			
Aspergillus niger	–	–	–
Bacillus subtilis	–	–	–
Candida albicans	–	–	–
Saccharomyces cerevisiae	–	–	–
Streptomyces murinus	–	–	–
Enzyme activity			
Nitrate reduction	–	–	–
Production of H_2S	–	+	+
Lipolysis	–	–	–
Pectin hydrolysis	–	–	–
Catalase	+	+	+
Melanine production	–	–	–
Degradation of			
Hypoxanthine	+	+	–
Elastin	–	–	–
Xanthine	–	+	–
Adenine	–	–	–
Strach	–	–	–
Elastin	–	–	–
Casein	–	–	–
Urea	–	–	–
Tyrosine	+	+	–
Gelatin	–	–	–
Esculin	+	ND	ND
Tween 80 (1%)	–	–	–
Pectin	–	–	–

Table 1. Continued

Unit Characters	Strain SCNU5^T	*T. tyrosinosolvens* DSM 44234^T	*T. pulmonis* DSM 44142^T
Growth in the presence of (%, w/v)			
Sodium chloride (4)	+	+	+
Sodium chloride (7)	+	+	+
Sodium chloride (10)	–	–	–
Sodium chloride (13)	–	–	–
Phenol (0.1)	+	+	+
Sodium azoid (0.01)	–	–	–
Sodium azoid (0.02)	–	–	–
Potassium tellurite (0.001)	+	+	+
Potassium tellurite (0.01)	+	+	+
Thallus acetate (0.001)	–	–	–
Thallus acetate (0.01)	–	–	–
Crystal violet (0.0001)	–	+	+

Table 2. Cellular fatty acid compositions (%) of strain SCNU5^T

Fatty acid	Composition (%)
14:0	2.79
15:0	1.06
16:1	Tr
16:0	38.72
15:0 iso 3-OH	Tr
17:0	Tr
18:1 cis 9	32.6
18:0	2.95
TBSA 18:0 10-methyl	11.39
20:1 cis 11	Tr
20:0	Tr

number of carbon atoms : number of double bonds
TBSA, tuberculostearic acid (10-methyloctadecanoic acid)
Tr, Trace amount (less than 1%)

resulted in two mycolate spots of α and α', as shown in *T. pulmonis* DSM 44142^T and *T. tyrosinosolvens* DSM 44234^T (data not shown). Gas chromatographic analyses of its fatty acid methyl esters revealed the presence of hexadecanoic acid (C16:0), octadecenoic acid (C18:1, cis9) and tuberculostearic acid (10 methyl octadecanoic acid) as major fatty acid methyl esters (Table 2). SCNU5^T contained glycolyl muramyl residue in the peptidoglycan (data not shown). SCNU5^T contained arabinose and galactose as whole cell sugars (whole cell sugar pattern A), and MK-8, MK-9, and MK-10. MK-9 was not hydrogenated (i.e., MK-9 (H_0)) and was the major component.

Molecular systematics

The G+C content of SCNU5^T was 68.1 mol %. The almost complete 16S rDNA sequence of strain SCNU5^T

was compared with the corresponding nucleotide sequences of mycolic acid containing actinomycetes (Fig. 1). The nucleotide sequence of strain SCNU5^T showed high similarity (99.66%) with both *T. pulmonis* 44142^T (4/1442) and *T. tyrosinosolvens* DSM 44234^T (5/1442). The levels of relatedness (binding rates) between the DNA of strain SCNU5^T and the DNAs of *T. pulmonis* 44142^T, *T. tyrosinosolvens* DSM 44234^T and *T. inchonensis* DSM 44067^T were, 54.1, 55.7 and 58.6%, respectively (Table 3).

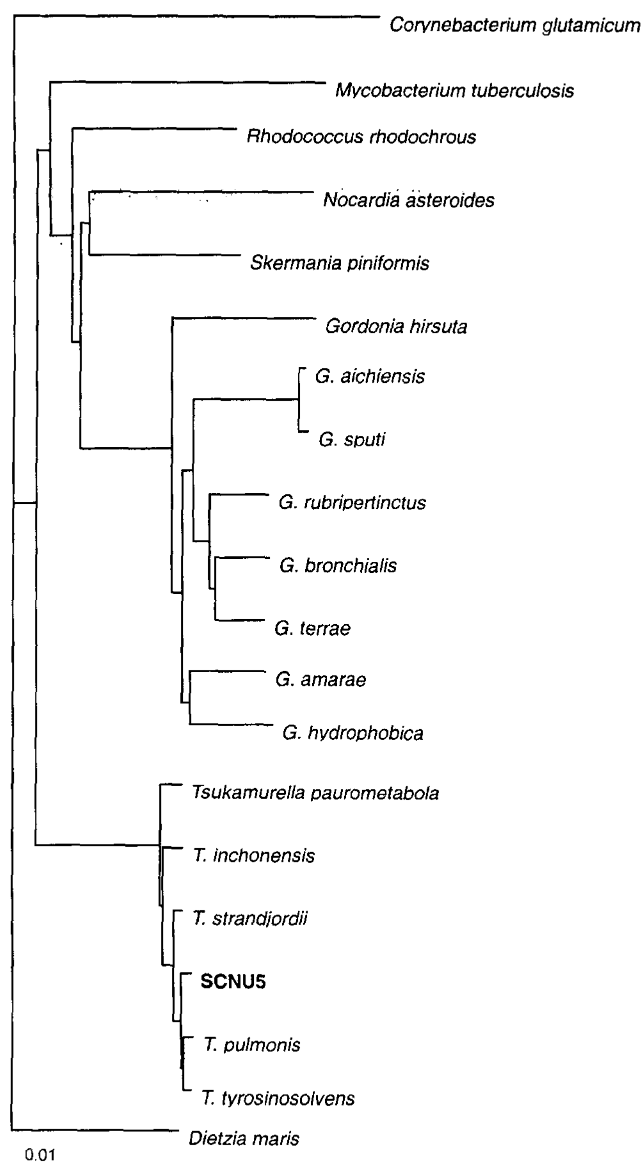

Fig. 1. A neighbor-joining tree based on the near complete 16S rDNA sequence of *Tsukamurella* isolate SCNU5^T, and representative species of a related taxa. The bar length corresponds to 0.01 substitutions per nucleotide position.

The physiological and chemotaxonomic characteristics of isolate SCNU5^T are compared with the characteristics of *T. pulmonis* DSM 44142^T and *T. tyrosinosolvens* DSM 44234^T in Table 1. Chemotaxonomically, strain SCNU5^T, *T. pulmonis* DSM 44142^T, and *T. tyrosinosolvens* DSM 44234^T are similar. All three strains contain galactose and arabinose as characteristic whole-cell sugars, and meso-diaminopimelic acid as a wall diamino acid (i.e., they are wall chemotype IV organisms). Moreover, all contain two mycolic acids corresponding to α and α'-mycolates, and all contain the glycolyl type of muramyl residue in their peptidoglycan. The fatty acid profiles of all three consist of saturated, monounsaturated and 10 methyl branched fatty acids, and all contain MK-9 as the major menaquinone. These chemotaxonomic similarities are supported by the observed high levels of 16S rDNA similarity between isolate SCNU5^T, *T. pulmonis* DSM 44142^T and *T. tyrosinosolvens* DSM 44234^T.

In contrast to the chemotaxonomic and nucleotide sequence similarities of isolate SCNU5^T, *T. pulmonis* DSM 44142^T and *T. tyrosinosolvens* DSM 44234^T, the results of physiological testing (Table 1) revealed clear differences between strain SCNU5^T and the other two strains.

Physiologically, isolate SCNU5^T differed in terms of; i) its inability to grow at 10°C or ii) on MacConkey agar, iii) its sensitivity to tobramycin, and its inability iv) to produce H_2S, v) to produce acid from sorbitol and mannose, and in terms of its inability to grow in the presence of crystal violet (0.0001%). In addition to these differences, strain SCNU5^T differs from *T. pulmonis* DSM 44142^T in terms of its ability i) to use maltose, inositol and melezitose as carbon sources, and ii) to degrade hypoxanthine and tyrosine. Finally, strain SCNU5^T differs from *T. tyrosinosolvens* DSM 44234^T in terms of its inability to; i) use benzoate and citrate as carbon sources, ii) degrade xanthine and iii) produce acid from xylitol.

Thus, we propose that strain SCNU5^T should be considered a new species of the genus *Tskamurella,* and has been deposited in the Korean Collection for Type Cultures (KCTC) as strain KCTC 9827^T.

Table 3. Levels of DNA binding for SCNU5^T, *T. pulmonis* DSM 44142^T, *T. tyrosinosolvens* DSM 44234^T and *T. inchonensis* DSM 44067^T

Microorganism	% Binding to		
	SCNU5	*T. pulmonis*	*T. tyrosinosolvens*
T. pulmonis DSM 44142^T	54.1		
T. tyrosinosolvens DSM 44234^T	55.7	63.4	
T. inchonensis DSM 44067^T	58.6	67.5	59.0

Description of *Tuskamurella sunchonensis* sp. nov.
Tuskamurella sunchonensis (sun.chon.ensis. M.L. n. *sunchon* Sunchon, a city in Korea; M.L. adj. *sunchonensis* of Sunchon, Korea; referring to the place where the organism was first isolated). Cells are aerobic, gram-positive and slightly acid-alcohol fast bacilli, and most cells appear as long rods. The cells do not form spores, capsules or aerial hyphae. Melanoid pigments are not produced, and the cells contain α and α' mycolic acids. Growth occurs at 28°C-37°C. The catalase reaction is positive, but not the lipase, pectinase or nitrate reductase reactions. The strain hydrolyzes hypoxanthine, tyrosine and esculine, but not casein, starch or urea. The strain utilizes fructose, sucrose, galactose and inositol as sole carbon sources, but not lactose, rhamnose or raffinose, and is sensitive to streptomycin, gentamicin or vancomycin, but resistant to both bacitracin and penicillin G. Acid is produced from glucose, trehalose, xylose, meletzitose, sucrose, fructose and maltose. The strain contain glycolyl muramyl residue. The major fatty acids present are hexadecanoic acid, octadecenoic acid and tuberculostearic acid, and the major isoprenoid quinone is MK-9. The strain contains arabinose and galactose as whole-cell sugars (i.e., wall chemotype IV). The G+C content of this strain was 68.1 mol %. The type strain is SCNU5^T (=KCTC 9827^T).

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