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Corynebacterium nuruki sp. nov., isolated from an alcohol fermentation starter

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A novel Gram-positive, strictly aerobic and non-motile bacterial strain, S6-4^T, was isolated from a Korean alcohol fermentation starter. Optimal growth occurred at 37 °C, at pH 8 and in 1 % (w/v) NaCl. The isolate was positive for oxidase and catalase. It assimilated various sugars and acids were produced from several carbohydrates. The major cell-wall sugars were galactose and arabinose. The major fatty acids of strain S6-4^T were $C_{16:0}$, $C_{17:1}\omega 9c$, $C_{18:1}\omega 9c$ and 10-methyl $C_{18:0}$ (tuberculostearic acid). The predominant isoprenoid quinone was menaquinone MK-9(H_2) and peptidoglycan amino acids were *meso*-diaminopimelic acid, alanine, glycine and glutamic acid. The strain contained mycolic acids. According to phylogenetic analysis based on 16S rRNA gene sequences, strain S6-4^T was most closely related to *Corynebacterium variabile* DSM 20132^T (98.1 % similarity). The genomic DNA G+C content of strain S6-4^T was 73.6 mol% and DNA–DNA hybridization values with related strains were below 33 ± 4 %. On the basis of phenotypic, genotypic and phylogenetic data, strain S6-4^T represents a novel species in the genus *Corynebacterium*, for which the name *Corynebacterium nuruki* sp. nov. is proposed; the type strain is S6-4^T (=KACC 15032^T =JCM 17162^T).

The genus *Corynebacterium* was introduced by Lehmann and Neumann in 1896 and, at the time of writing, comprised over 100 species. Members of this genus can be distinguished from those of other genera by their chemotaxonomic characteristics, e.g. they contain a directly cross-linked *meso*-diaminopimelic acid-containing type A1γ peptidoglycan moiety (Schleifer & Kandler, 1972), fatty acids such as tuberculostearic acid (TBSA) (Collins *et al.*, 1982) and MK-9(H_2) as a predominant menaquinone type (Collins *et al.*, 1985). Although many of the strains of this genus are derived from human clinical samples or animal specimens, strain S6-4^T, described in this study, was isolated from food-related material. A coryneform bacterium was isolated from *nuruk*, which is an alcohol fermentation starter used as an amylolytic substance in the manufacture of traditional Korean rice wine. *Nuruk* is a mixture of grains and various micro-organisms including mould, yeast and bacteria. These components execute fermentation of rice and make an alcoholic beverage named *makgeolli* (Aidoo *et al.*, 2006). An antibiotic-containing medium, which limits the growth of eukaryotes, was used for isolation of bacteria. Based on evidence from physiological, biochemical and genotypic investigations, it is proposed that strain S6-4^T belongs to a novel species of the genus *Corynebacterium*.

Abbreviation: TBSA, tuberculostearic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S6-4^T is HM165487.

A supplementary figure is available with the online version of this paper.

Strain S6-4^T was isolated from *nuruk* by spreading and incubating serially diluted samples at 30 °C on R2A agar (Difco) medium containing 100 mg cycloheximide l^{-1}, which is an inhibitor of protein biosynthesis in eukaryotes. The isolate was repeatedly subcultured to obtain a pure culture. All physiological and biochemical studies were performed in triplicate with the isolate cultured at 30 °C for 48 h on peptone-yeast-brain-heart infusion (PY-BHI) broth or agar medium (Takeuchi *et al.*, 1999). Ranges and optimal conditions for growth were determined at 4, 10, 25, 27, 30, 37, 45 and 65 °C and at pH 5.0–10.0 at unit intervals in PY-BHI medium. NaCl tolerance was determined by assessing growth in PY-BHI medium containing 0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 % (w/v) NaCl. After incubation for 24 h, 48 h and 7 days, turbidity of cultures was measured at 600 nm using a spectrophotometer (SYNERGY MX; BioTek). Growth of the isolate under anaerobic conditions was determined by incubation for 7 days in an anaerobic chamber filled with 90 % N_2, 5 % H_2 and 5 % CO_2. Gram staining was accomplished using a Gram staining kit (bioMérieux) and observed on a light microscope (ECLIPSE 50*i*; Nikon). Cellular motility was examined using semi-solid agar (Tittsler & Sandholzer, 1936).

Strain S6-4^T was Gram-positive, non-motile, and grew at 10–45 °C (optimum 37 °C), at pH 6.0–9.0 (optimum

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pH 8.0) and in the presence of 0–10 % (w/v) NaCl (optimum 1 %). Growth under anaerobic conditions was not observed over the 7 day incubation period. The isolate grew on nutrient agar (NA; Difco), tryptic soy agar (TSA; Bacto), Columbia agar (BBL) supplemented with 5 % horse blood and Corynebacterium agar (DSM medium no. 53) containing 1 % tryptone, 0.5 % yeast extract, 0.5 % glucose and 0.5 % NaCl (pH 7.2).

The 16S rRNA gene sequence of the isolate was amplified by colony PCR with PCR Pre-Mix (SolGent) and two universal primers for bacteria (8F, 1492R) (Baker *et al.*, 2003). The PCR product was purified using the QIAquick PCR Purification kit and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. Reaction mixtures were analysed using an automated DNA analyser system (PRISM 3730XL DNA analyser; Applied Biosystems). Fragments of the 16S rRNA gene sequence were assembled with SeqMan (DNASTAR) to give an almost full-length 16S rRNA gene sequence. The assembled 16S rRNA gene sequence (1515 bp) was processed by the EzTaxon server (Chun *et al.*, 2007) and compared with those of all species of the genus *Corynebacterium* with validly published names in the EzTaxon database. Results showed that strain S6-4^{T} had the highest pairwise similarities to *Corynebacterium variabile* DSM 20132^{T} and *Corynebacterium terpenotabidum* IFO 14764^{T} (98.1 % and 97.0 %, respectively). 16S rRNA gene sequences of strain S6-4^{T} and strains of closely related species based on EzTaxon server data were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic relationships with species of the genus *Corynebacterium* were determined using MEGA4 (Tamura *et al.*, 2007). Using neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods, phylogenetic consensus trees were reconstructed from 1000, 1000 and 300 random bootstrap replicates, respectively. In the reconstructed consensus trees, strain S6-4^{T} formed a monophyletic cluster with *C. variabile* DSM 20132^{T} and *C. terpenotabidum* IFO 14764^{T} (Fig. 1).

Sole carbon source assimilation and acid production were screened using API 50CH with AUX medium and API 50CHB test strips (bioMérieux) and several biochemical properties, including enzyme activities and fermentation ability, were investigated using API Coryne test strips (bioMérieux), according to the manufacturer's instructions. Reactions were read after 72 h of incubation at 30 °C. *C. variabile* DSM 20132^{T} (=KCTC 9102^{T}) and *C. terpenotabidum* IFO 14764^{T} (=JCM 10555^{T}), obtained from the Korean Collection for Type Cultures (KCTC) and the Japan Collection of Microorganisms (JCM), respectively, were used as reference strains. Data for strain S6-4^{T} and the two reference strains, the phylogenetically most closely related strains based on 16S rRNA gene sequence analysis, are given in Table 1. Catalase and oxidase activities were examined by observing bubble production in 3 % (v/v) hydrogen peroxide solution and using 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively.

The isolate was catalase- and oxidase-positive. Strain S6-4^{T} had a number of features that enabled it to be distinguished from the reference strains, i.e. urease activity (API Coryne), acid production from D-ribose, D-galactose and methyl α-D-glucoside (API 50CHB), assimilation of D-adonitol,

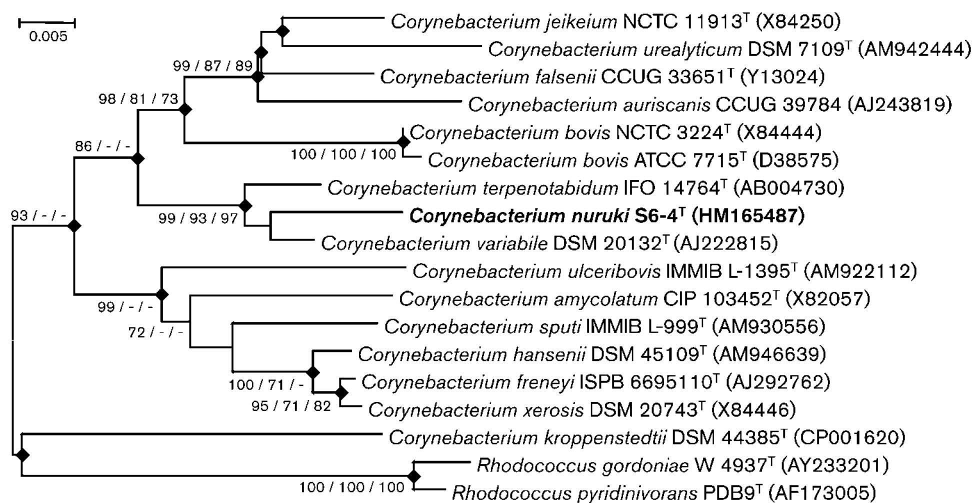

Fig. 1. Phylogenetic tree, generated by the neighbour-joining method, based on 16S rRNA gene sequence analysis showing the positions of strain S6-4^{T} and other type strains of the genus *Corynebacterium*. Numbers at nodes indicate bootstrap values (>70 %) based on neighbour-joining, maximum-parsimony and maximum-likelihood algorithms as percentages of 1000, 1000 and 300 replicates, respectively; dashes indicate bootstrap values of 70 % or less. Filled diamonds show common branches recovered in all three algorithms. Bar, 0.005 substitutions per nucleotide.

Table 1. Biochemical characteristics of strain S6-4^T and its closest phylogenetic relatives

Strains: 1, *Corynebacterium nuruki* S6-4^T sp. nov.; 2, *C. variabile* DSM 20132^T; 3, *C. terpenotabidum* IFO 14764^T. Data for enzyme activities, carbon source assimilation and acid production were obtained from API Coryne, API 50CH and API 50CHB, respectively. All data were obtained from this study. All strains were positive for pyrazinamidase (API Coryne), for D-glucose, D-fructose, D-mannose, turanose and gluconate assimilation (API 50CH) and for acid production from D-glucose, D-fructose, D-mannose and 5-ketogluconate (API 50CHB).

Characteristic	1	2	3
Oxidase	+	+	−
Enzyme activity			
Alkaline phosphatase	+	−	+
α-Glucosidase	−	−	+
Urease	−	+	+
Assimilation of:			
D-Ribose	+	−	+
D-Xylose	+	+	−
L-Xylose	+	+	−
D-Adonitol	+	−	−
D-Galactose	+	−	−
L-Sorbose	+	+	−
L-Rhamnose	+	−	−
Dulcitol	+	−	−
Inositol	+	−	−
D-Mannitol	+	−	+
D-Sorbitol	+	−	+
Methyl α-D-glucoside	+	−	+
N-Acetylglucosamine	+	−	+
Amygdalin	+	−	−
Aesculin	+	+	−
Salicin	−	+	+
Cellobiose	−	+	−
Lactose	+	−	+
Sucrose	−	−	+
Trehalose	+	−	+
Inulin	+	−	+
Melezitose	+	−	−
Raffinose	−	−	+
Glycogen	−	−	+
Gentiobiose	+	−	+
D-Lyxose	+	−	+
D-Tagatose	−	+	−
D-Fucose	−	−	+
L-Fucose	−	−	+
D-Arabitol	−	+	+
L-Arabitol	−	−	+
2-Ketogluconate	−	−	+
5-Ketogluconate	+	−	−
Acid production from:			
Glycerol	−	+	−
D-Ribose	+	−	−
D-Galactose	+	−	−
D-Mannitol	−	+	−
Methyl α-D-glucoside	+	−	−
Arbutin	−	+	−
Aesculin	−	+	−
Salicin	−	+	+
Cellobiose	−	−	+
D-Arabitol	−	+	−
Gluconate	−	+	−

D-galactose, L-rhamnose, dulcitol, inositol, amygdalin, salicin, melezitose, D-arabitol and 5-ketogluconate (API 50CH), and oxidase activity.

For analyses of cell chemistry, including cell-wall sugars, fatty acids, menaquinones, amino acids and mycolic acids, cells of strain S6-4^T and reference strains cultured on PY-BHI agar medium at 30 °C for 48 h were harvested. Cell-wall sugars were determined by the method of Schleifer & Kandler (1972) with galactose, glucose, mannose, arabinose, xylose and rhamnose as standard sugars (Schön & Groth, 2006). Fatty acids were extracted as described by the Sherlock Microbial Identification System (MIDI, 1999), analysed by GC (Hewlett Packard 6890) and identified with the Microbial Identification software package (Sasser, 1990). For analysis of menaquinone types, menaquinones were extracted from freeze-dried cells with methanol/diisopropyl ether (2.5 : 1, v/v) and analysed by TLC using the protocol of Hiraishi *et al.* (1996). The amino acid composition of the cell-wall peptidoglycan was analysed using one-dimensional TLC on cellulose sheets (Bousfield *et al.*, 1985) with LL- and *meso*-diaminopimelic acid, 2,4-diaminobutyric acid, alanine, glutamic acid and glycine as standard amino acids. Mycolic acids were extracted, purified by the method of Yano & Saito (1972) and detected by TLC as described by Goodfellow *et al.* (1976).

Strain S6-4^T possessed arabinose and galactose as cell-wall sugars, menaquinone MK-9(H_2) as predominant menaquinone, with MK-9, MK-8(H_2) and MK-8 as minor components, mycolic acids, and *meso*-diaminopimelic acid, alanine and glutamic acid in the peptidoglycan. The cellular fatty acids of the isolate were $C_{16:0}$ (51.3 %), $C_{17:1}\omega9c$ (20.0 %), $C_{18:1}\omega9c$ (10.2 %), 10-methyl $C_{18:0}$ (TBSA; 8.2 %), $C_{14:0}$ (4.5 %), iso-$C_{18:1}$ H (1.1 %), iso-$C_{19:1}$ I (0.8 %) and iso-$C_{17:0}$ 3-OH (0.7 %). Strain S6-4^T belongs to the genus *Corynebacterium* based on its chemotaxonomic features, i.e. its cell-wall sugar composition, directly cross-linked *meso*-diaminopimelic acid-containing type (A1γ) (Schleifer & Kandler, 1972) cell wall hydrolysates and cellular fatty acids (Collins *et al.*, 1982), menaquinone types (Collins *et al.*, 1985) and the presence of mycolic acids.

Chromosomal DNA was extracted using the method described by Rochelle *et al.* (1992) with cell lysis achieved by bead beating. The DNA G+C content of the entire genome was determined by a fluorimetric method using SYBR Gold I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). The genomic DNA G+C content of

Table 2. Levels of DNA–DNA hybridization among strain S6-4^T and reference strains

Strain	DNA–DNA hybridization (%) with:		
	S6-4^T	DSM 20132^T	IFO 14764^T
C. nuruki S6-4^T	100 ± 0	42 ± 3	45 ± 5
C. variabile DSM 20132^T	33 ± 4	100 ± 0	27 ± 4
C. terpenotabidum IFO 14764^T	17 ± 5	41 ± 4	100 ± 0

the isolate was 73.6 mol%. DNA–DNA hybridization was performed using genome-spotted microarrays (Bae *et al.*, 2005; Chang *et al.*, 2008) to determine the genetic relatedness between the isolate and the reference species. The genomic DNA of *Escherichia coli* K-12 was used as a negative control. Cy5-dUTP labelled target DNA (1 μg) was mixed with a hybridization solution containing 50 % formamide, 3× SSC, 1.25 μg unlabelled herring sperm DNA and 0.3 % SDS; 7 μl mixture was hybridized with probe DNA on a microarray slide. The microarray slide was placed into a hybridization chamber, boiled for 5 min to denature the hybridization solution and plunged immediately into the 37 °C water bath for overnight hybridization. The microarray slide was scanned with a GenePix 400A (Axon instruments) microarray scanner (Supplementary Fig. S1, available in IJSEM Online) and the signal-to-noise ratio of each probe was calculated with the formula reported previously (Loy *et al.*, 2005). According to prior research of Chang *et al.* (2008), this DNA–DNA hybridization method gives improved accuracy and precision compared with other traditional methods using a microplate (Ezaki *et al.*, 1989) or a nylon membrane (Kafatos *et al.*, 1979). DNA–DNA hybridization values between the isolate and reference species were as follows: 33 ± 4 % with *C. variabile* DSM 20132^T and 17 ± 5 % with *C. terpenotabidum* IFO 14764^T (Table 2). DNA–DNA relatedness values below a threshold of 70 % (Wayne *et al.*, 1987) indicate that strain S6-4^T represents a distinct genomic species.

Based on its phenotypic, phylogenetic and genotypic characteristics, it is clear that strain S6-4^T represents a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium nuruki* sp. nov. is proposed.

Description of *Corynebacterium nuruki* sp. nov.

Corynebacterium nuruki (nu.ru′ki. N.L. n. *nurukum* nuruk, Korean name of an amylolytic starter used for Korean traditional rice wine; N.L. gen. n. *nuruki* of/from nuruk).

Cells are Gram-positive, strictly aerobic, irregular rod-shaped and non-motile. Colonies on PY-BHI agar medium are circular, 0.5–1.0 mm in diameter, smooth, opaque, convex, slightly viscous and cream-coloured after 48 h at 30 °C. Growth occurs at temperatures of 10–45 °C, in the presence of 0–10 % (w/v) NaCl and at pH 6.0–9.0. Optimal growth conditions are 37 °C, 1 % NaCl and pH 8. Catalase and oxidase are produced. Positive for alkaline phosphatase, but negative for nitrate reductase, pyrrolidonyl arylamidase, β-glucuronidase, β-galactosidase, *N*-acetyl-β-glucosaminidase and β-glucosidase (API Coryne). Assimilates D-ribose, DL-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, aesculin, lactose, inulin, melezitose, gentiobiose, turanose, D-lyxose, gluconate and 5-ketogluconate (API 50CH). Acid is produced from D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside and 5-ketogluconate (API 50CHB). The major cell-wall sugars are arabinose and galactose. The peptidoglycan is directly cross-linked *meso*-diaminopimelic acid-containing type A1γ, consisting of *meso*-diaminopimelic acid, alanine and glutamic acid. The major fatty acids are $C_{16:0}$, $C_{17:1}\omega 9c$, $C_{18:1}\omega 9c$ and TBSA 10-methyl $C_{18:0}$. The predominant menaquinone is MK-9(H_2) and mycolic acids are present. The DNA G+C content of the type strain is 73.6 mol%.

The type strain is S6-4^T (=KACC 15032^T =JCM 17162^T), isolated from *nuruk*, an alcohol fermentation starter of traditional Korean wine.

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