Corynebacterium doosanense sp. nov., isolated from activated sludge

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The taxonomic position of a Gram-positive, non-motile, non-spore-forming coryneform, isolated from activated sludge and designated strain CAU 212^T, was investigated using a polyphasic approach. Cellular morphology, biochemical tests and chemotaxonomic investigations revealed that strain CAU 212^T had the characteristics of the genus *Corynebacterium*. Comparative 16S rRNA gene sequence analysis showed that the organism formed a hitherto-unknown subline within the genus *Corynebacterium*. Sequence divergence values of more than 4.3 % from recognized *Corynebacterium* species, together with phenotypic differences, showed that the bacterium represents a previously unrecognized member of the genus *Corynebacterium*, for which the name *Corynebacterium doosanense* sp. nov. is proposed. The type strain is CAU 212^T (=KCTC 19568^T=CCUG 57284^T).

The genus *Corynebacterium* was established by Lehmann & Neumann (1896) and represents one of the largest groups within the *Actinobacteria*. At the time of writing, *Corynebacterium* comprised 95 recognized taxa. In recent years, many of the recognized species within this genus originated from clinical specimens of humans (Hall *et al.*, 2003; Renaud *et al.*, 2007; Yassin, 2007; Yassin & Siering, 2008), animals (Goyache *et al.*, 2003a, b; Fernández-Garayzábal *et al.*, 2003, 2004; Collins *et al.*, 2004; Vela *et al.*, 2006) and environments (Yassin *et al.*, 2003; Chen *et al.*, 2004; Feurer *et al.*, 2004). Until now, however, there have been relatively few reports of corynebacteria from activated sludge. During the course of routine screening of bacteria for industrial purposes, a *Corynebacterium*-like strain, designated CAU 212^T, was isolated from activated sludge from the wastewater treatment plant in Yeongdeuk-gun, Republic of Korea.

The isolation procedure for strain CAU 212^T followed that of Gordon & Mihm (1962) by using glucose-yeast extract agar (GYEA; comprising per litre: 10 g yeast extract, 10 g glucose and 15 g agar) supplemented with 50 mg cycloheximide l⁻¹ and 20 mg nalidixic acid l⁻¹. An activated sludge sample was diluted with sterilized distilled water and appropriate dilutions were spread onto the GYEA medium and incubated aerobically for 3 days at 30 °C. An opaque, yellow, low-convex bacterial colony with a diameter of 1–2 mm was subcultured on sheep blood agar plates (Asan

Pharm Co.). The pure culture of CAU 212^T was preserved in 25 % (v/v) glycerol at –70 °C. The cell morphology of colonies was observed at different incubation times, every 24 h for 3 days, with a microscope following Gram staining.

Strain CAU 212^T was characterized biochemically using the API Coryne, API 20 Strep and API ZYM kits (bioMérieux) according to the manufacturer's instructions. The Christie–Atkins–Munch–Petersen (CAMP) test with *Staphylococcus aureus* was performed as described by von Graevenitz & Funke (1996). Cell-wall murein was prepared by mechanical disruption of cells and complete acid hydrolysates were analysed as described by Schleifer & Kandler (1972). Cellular fatty acid methyl esters were extracted by acid methanolysis (Minnikin *et al.*, 1980) and analysed by using a Hewlett Packard series II gas chromatograph model 5890A equipped with a 5 % phenyl methyl silicon-fused silica capillary column (HP 19091B-102). The presence of mycolic acid was investigated by GLC analysis of trimethylsilylated derivatives (Klatte *et al.*, 1994).

Genomic DNA of strain CAU 212^T was isolated and purified by using the method of Marmur (1961). The DNA G+C content was measured by using the thermal denaturation method (Marmur & Doty, 1962). PCR amplification and sequencing of the 16S rRNA gene were carried out following established procedures (Nam *et al.*, 2004). The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple alignments

Abbreviation: CAMP, Christie–Atkins–Munch–Petersen.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain CAU 212^T is EU998655.

with sequences of a broad selection of corynebacteria and calculations of sequence similarity levels were carried out by using CLUSTAL X (Thompson *et al.*, 1997) and the EzTaxon server (Chun *et al.*, 2007). A phylogenetic tree was constructed using the neighbour-joining algorithm (Saitou & Nei, 1987) from the PHYLIP suite of programs (Felsenstein, 1989). Evolutionary distances for the neighbour-joining analysis were based on the correction of Jukes & Cantor (1969). Tree topology was evaluated by using the bootstrap resampling method with 1000 replicates (Felsenstein, 1985) of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package.

Colonies of strain CAU 212^T on GYEA plates growing under aerobic conditions at 30 °C for 3 days were yellow, opaque, low-convex and approximately 1–2 mm in diameter. On sheep blood agar after 48 h of incubation, colonies were small (about 1 mm in diameter), yellow, opaque and non-haemolytic. Microscopic examination of the cells showed Gram-positive, non-motile, irregular and club-shaped rods, a typical coryneform morphology. Mycelia and spores were not observed. Cells were catalase- and oxidase-positive. API Coryne, API 20 Strep and API ZYM test strips provided adequate phenotypic data to describe the novel isolate and a detailed description of the key phenotypic characteristics of the isolate is given in Table 1 and in the species description.

Using the API Coryne system, positive results were obtained for the production of pyrazinamidase, production of acid from glucose and reduction of nitrate. The API Coryne code for strain CAU 212^T was 3000104 and generated poor confidence levels towards *Corynebacterium accolens*, *C. striatum/amycolatum* and *C. argentoratense* when compared with API Coryne database version 3. Results from the API 20 Strep system were positive for sodium pyruvate and hippuric acid hydrolysis. The API

ZYM test detected esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. The CAMP test was negative. An examination of acid hydrolysate of cell-wall murein revealed the presence of *meso*-diaminopimelic acid as the dibasic amino acid. TLC analysis of whole-cell hydrolysates detected mainly galactose and arabinose. Short-chain mycolic acids were also detected. Predominant fatty acids corresponded to C_{14:0} (0.3 %), C_{16:1ω9c} (1.1 %), C_{16:0} (18.8 %), C_{18:1ω9c} (67.0 %) and C_{18:0} (9.9 %). The major long-chain cellular fatty acids of the organism were C_{12:0} (3 %), C_{14:0} (4 %), C_{16:0} (28 %), C_{18:0} (25 %), C_{18:1ω9c} (28 %), C_{18:1ω7c} (1 %) and C_{20:0} (2 %). Small amounts of short-chain mycolic acids (C₂₂–32) were also detected. The DNA G+C content of strain CAU 212^T was 53.5 mol%.

The nearly complete 16S rRNA gene sequence of strain CAU 212^T (1494 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. A phylogenetic tree, based on 16S rRNA gene sequence data from strain CAU 212^T and corresponding sequences from the type strains of recognized corynebacteria as well as *Tsukamurella paurometabola* DSM 20162^T, was constructed according to the neighbour-joining algorithm (Fig. 1). The genus *Corynebacterium* embraces a very diverse range of organisms and it is recognized that the genus is not monophyletic and comprises several distinct 16S rRNA gene lineages. The tree topology, supported by high bootstrap values, clearly separated strain CAU 212^T within the genus *Corynebacterium*. It is evident from our 16S rRNA gene sequence study that strain CAU 212^T is a distinct member of the subclade containing *Corynebacterium hansenii* C-138^T, *C. xerosis* ATCC 373^T, *C. freneyi* ISPB 6695110^T and *C. halotolerans* YIM 70093^T within the phylogenetic tree. Pairwise similarity values ranged between 95.7 % (*C. hansenii* CIP 108444^T) and 90.3 % (*C. hoagii* ATCC 7005^T). High sequence divergence values (>4.3 %) with

Table 1. Differential phenotypic properties of strain CAU 212^T and its closest phylogenetically related species

Strains: 1, CAU 212^T (*C. doosanense* sp. nov.); 2, *C. hansenii* CIP 108444^T; 3, *C. singulare* CCUG 37330^T; 4, *C. minutissimum* NCTC 10288^T; 5, *C. xerosis* ATCC 373^T; 6, *C. freneyi* ISPB 6695110^T; 7, *C. halotolerans* YIM 70093^T. All strains produce acid from glucose. All data are from this study. +, Positive; –, negative; v, variable.

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	+	–	–	–	v	–	+
Hydrolysis of:							
Gelatin	–	–	–	–	–	–	+
Urea	–	–	+	v	–	–	+
Acid from:							
Ribose	–	+	–	–	+	+	–
Maltose	–	+	+	+	+	+	–
Activity for:							
β-Glucuronidase	–	–	–	–	v	–	+
α-Glucosidase	–	–	–	–	+	+	–
Pyrazinamidase	+	+	+	+	+	+	–

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 212^T and members of the genus *Corynebacterium*. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. *Tsukamurella paurometabola* DSM 20162^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

recognized members of this genus clearly indicate that strain CAU 212^T represents a novel species of the genus *Corynebacterium* (Stackebrandt & Goebel, 1994).

The phenotypic and chemotaxonomic data, together with the 16S rRNA gene sequence data, provide sufficient evidence to support the proposal that strain CAU 212^T represents a novel species, *Corynebacterium doosanense* sp. nov.

Description of *Corynebacterium doosanense* sp. nov.

Corynebacterium doosanense (doo.san.en'se. N.L. neut. adj. *doosanense* belonging to Doosan, named after the Doosan group, the Foundation of Chung-Ang University where the taxonomic studies on this species were performed).

Cells are aerobic, Gram-positive, non-motile, non-spore-forming, irregular and club-shaped rods, 0.8–1.0 × 1.0–1.2 µm in size. Colonies (1–2 mm in diameter) are yellow and low-convex on GYEA media and yellow, opaque and low-convex on sheep blood agar after 48 h incubation at 30 °C. Catalase, oxidase, reduction of nitrate and hydrolysis of hippuric acid are positive and activities are detected for sodium pyruvate, pyrazinamidase, esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. No activity is detected for pyrrolidonyl arylamidase,

alkaline phosphatase, esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, α-chymotrypsin, β-glucuronidase, α- and β-galactosidase, α- and β-glucosidase, α-mannosidase, α-fucosidase, *N*-acetyl-β-glucosaminidase, leucine aminopeptidase, urease or hydrolysis of gelatin and aesculin. Acid production occurs only from glucose. Cell wall contains *meso*-diaminopimelic acid. Whole-cell hydrolysates contain mainly galactose and arabinose. Mycolic acids are detected. The DNA G+C content of the type strain is 53.5 mol%.

The type strain is CAU 212^T (=KCTC 19568^T=CCUG 57284^T), isolated from activated sludge taken from a wastewater treatment plant in Yeongdeuk-gun, Republic of Korea.

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References

- Chen, H.-H., Li, W.-J., Tang, S.-K., Kroppenstedt, R. M., Stackebrandt, E., Xu, L.-H. & Jiang, C.-L. (2004). *Corynebacterium halotolerans* sp. nov.,

- isolated from saline soil in the west of China. *Int J Syst Evol Microbiol* 54, 779–782.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* 57, 2259–2261.
- Collins, M. D., Hoyles, L., Foster, G. & Falsen, E. (2004). *Corynebacterium caspium* sp. nov., from a Caspian seal (*Phoca caspica*). *Int J Syst Evol Microbiol* 54, 925–928.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* 5, 164–166.
- Fernández-Garayzábal, J. F., Egido, R., Vela, A. I., Briones, V., Collins, M. D., Mateos, A., Hutson, R. A., Domínguez, L. & Goyache, J. (2003). Isolation of *Corynebacterium falsenii* and description of *Corynebacterium aquilae* sp. nov., from eagles. *Int J Syst Evol Microbiol* 53, 1135–1138.
- Fernández-Garayzábal, J. F., Vela, A. I., Egido, R., Hutson, R. A., Lanzarot, M. P., Fernández-García, M. & Collins, M. D. (2004). *Corynebacterium ciconiae* sp. nov., isolated from the trachea of black storks (*Ciconia nigra*). *Int J Syst Evol Microbiol* 54, 2191–2195.
- Feurer, C., Clermont, D., Bimet, F., Candréa, A., Jackson, M., Glaser, P., Bizet, C. & Dauga, C. (2004). Taxonomic characterization of nine strains isolated from clinical and environmental specimens, and proposal of *Corynebacterium tuberculoearicum* sp. nov. *Int J Syst Evol Microbiol* 54, 1055–1061.
- Gordon, R. E. & Mihm, J. M. (1962). Identification of *Nocardia caviae* (Erikson) nov. comb. *Ann N Y Acad Sci* 98, 628–636.
- Goyache, J., Ballesteros, C., Vela, A. I., Collins, M. D., Briones, V., Hutson, R. A., Potti, J., García-Borboroglu, P., Domínguez, L. & Fernández-Garayzábal, J. F. (2003a). *Corynebacterium sphenisci* sp. nov., isolated from wild penguins. *Int J Syst Evol Microbiol* 53, 1009–1012.
- Goyache, J., Vela, A. I., Collins, M. D., Ballesteros, C., Briones, V., Moreno, J., Yorío, P., Domínguez, L., Hutson, R. & Fernández-Garayzábal, J. F. (2003b). *Corynebacterium spheniscorum* sp. nov., isolated from the cloacae of wild penguins. *Int J Syst Evol Microbiol* 53, 43–46.
- Hall, V., Collins, M. D., Hutson, R. A., Lawson, P. A., Falsen, E. & Duerden, B. I. (2003). *Corynebacterium atypicum* sp. nov., from a human clinical source, does not contain corynomycolic acids. *Int J Syst Evol Microbiol* 53, 1065–1068.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. edited by H. H. Munro. New York: Academic Press.
- Klatte, S., Kroppenstedt, R. M. & Rainey, F. A. (1994). *Rhodococcus opacus* sp. nov., an unusual nutritionally versatile *Rhodococcus*-species. *Syst Appl Microbiol* 14, 355–360.
- Lehmann, K. B. & Neumann, R. (1896). *Atlas und Grundriss der Bakteriologie und Lehrbuch der Speziellen Bakteriologischen Diagnostik*. Munich: J. F. Lehmann (in German).
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 3, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 5, 109–118.
- Minnikin, D. E., Hutchinson, I. G., Caldicott, A. B. & Goodfellow, M. (1980). Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. *J Chromatogr A* 188, 221–233.
- Nam, S. W., Kim, W., Chun, J. & Goodfellow, M. (2004). *Tsukamurella pseudospumae* sp. nov., a novel actinomycete isolated from activated sludge foam. *Int J Syst Evol Microbiol* 54, 1209–1212.
- Renaud, F. N. R., Coustumier, A. L., Wilhem, N., Aubel, D., Riegel, P., Bollet, C. & Freney, J. (2007). *Corynebacterium hansenii* sp. nov., an α -glucosidase-negative bacterium related to *Corynebacterium xerosis*. *Int J Syst Evol Microbiol* 57, 1113–1116.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–425.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 36, 407–477.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44, 846–849.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876–4882.
- Vela, A. I., Gracia, E., Fernández, A., Domínguez, L. & Fernández-Garayzábal, J. F. (2006). Isolation of *Corynebacterium xerosis* from animal clinical specimens. *J Clin Microbiol* 44, 2242–2243.
- von Graevenitz, A. & Funke, G. (1996). An identification scheme for rapidly and aerobically growing gram-positive rods. *Zentralbl Bakt* 284, 246–254.
- Yassin, A. F. (2007). *Corynebacterium ureicelerivorans* sp. nov., a lipophilic bacterium isolated from blood culture. *Int J Syst Evol Microbiol* 57, 1200–1203.
- Yassin, A. F. & Siering, C. (2008). *Corynebacterium sputi* sp. nov., isolated from the sputum of a patient with pneumonia. *Int J Syst Evol Microbiol* 58, 2876–2879.
- Yassin, A. F., Kroppenstedt, R. M. & Ludwig, W. (2003). *Corynebacterium glaucum* sp. nov. *Int J Syst Evol Microbiol* 53, 705–709.