Corynebacterium epidermidicanis sp. nov., isolated from skin of a dog

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A Gram-stain-positive, pleomorphic, oxidase-negative, non-motile isolate from the skin of a dog, designated strain 410^T, was subjected to comprehensive taxonomic characterization. Comparison of the 16S rRNA gene sequences revealed that the novel isolate showed highest similarities to the type strains of *Corynebacterium humireducens*, *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* (96.1–96.8%). The quinone system consisted predominantly of MK-8(H₂) and MK-9(H₂). The polar lipid profile of strain 410^T contained the major compounds diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-inositol, two unidentified phospholipids and four unidentified glycolipids. The polyamine pattern was composed of the major amines spermidine and spermine. In the fatty acid profile, predominantly straight-chain, saturated and mono-unsaturated fatty acids were detected (C_{18:1}ω₉C, C_{16:1}ω₇C, C_{16:0}). These chemotaxonomic traits are in agreement with those reported for representatives of the genus *Corynebacterium*. Strain 410^T tested negative for diphtheria toxin. Physiological properties as well as unique traits in the polar lipid profile could be used to distinguish strain 410^T from the most closely related species. These data suggest that strain 410^T represents a novel species of the genus *Corynebacterium*, for which we propose the name *Corynebacterium epidermidicanis* sp. nov. The type strain is 410^T (=DSM 45586^T=LMG 26322^T=CCUG 60915^T).

Currently, the genus *Corynebacterium* comprises more than 80 recognized species of which six were proposed in 2010: *Corynebacterium canis* (Funke *et al.*, 2010a), *Corynebacterium marinum* (Du *et al.*, 2010), *Corynebacterium mustelae* (Funke *et al.*, 2010b), *Corynebacterium pilbarensis* (Aravena-Roman *et al.*, 2010), *Corynebacterium pyruviciproducens* (Tong *et al.*, 2010) and *Corynebacterium stationis* (Bernard *et al.*, 2010). The majority of representatives of the genus contain short chain mycolic acids (22–38 carbons; Collins *et al.*, 1982a) but mycolic acids are absent in five species: *Corynebacterium amycolatum*, *Corynebacterium atypicum*, *Corynebacterium caspium*, *Corynebacterium ciconiae* and *Corynebacterium kroppenstedtii* (Collins *et al.*, 1988, 1998, 2004; Fernández-Garayzábal *et al.*, 2004; Hall *et al.*, 2003). The quinone

system is composed of dihydrogenated menaquinones with eight or nine isoprenoid units in the side-chain [MK-8(H₂), MK-9(H₂) or a mixture of both] (Collins & Jones, 1981). The fatty acid profiles consist predominantly of straight-chain saturated and mono-unsaturated fatty acids but some species also contain tuberculostearic acid (Collins *et al.*, 1982b). The peptidoglycan type is A1γ with the diagnostic diamino acid *meso*-diaminopimelic acid (Schleifer & Kandler, 1972). Although several species of the genus are well-known pathogens of humans and animals, including mammals and birds, species have been also isolated from other sources such as activated sludge and soil.

During the course of a study dealing with the classification of bacterial strains considered to be commensals that were isolated from primary cultures of veterinary specimens taken from individuals with different diseases, strain 410^T attracted our attention. Strain 410^T was isolated from the

Abbreviation: NP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 410^T is FR874223.

skin of a dog with pruritus after primary cultivation on blood agar. Colony counts of this strain were significant among the predominant colonies of β -haemolytic streptococci and *Proteus* strains (identified during routine diagnosis). The strain was grown aerobically on PYE agar (0.3 % peptone from casein, 0.3 % yeast extract, 1.5 % agar, pH 7.2) or the corresponding broth medium at 28 °C. Anaerobic growth was tested in an anaerobic jar using AnaeroGen (Oxoid). Gram-behaviour was examined by staining as described by Murray *et al.* (1994) following the Hucker method and KOH-lysis test as described by Moaledj (1986). Cell morphology was observed under an Olympus Provis AX70 microscope at $\times 1000$ magnification. In both tests, Gram-behaviour was positive. The cells exhibited a pleomorphic shape (coccoid and rod-shaped). The presence of catalase was tested using 3 % (v/v) H₂O₂; the test revealed the presence of catalase. Lipophilic behaviour was tested on brain heart infusion agar supplemented with 1 % (v/v) Tween 80 and strain 410^T was shown not to be lipophilic. The CAMP reaction was tested on Columbia agar supplemented with 5 % sheep blood with *Staphylococcus aureus* ATCC 25923; the CAMP reaction with strain 410^T was positive. Biochemical identification was performed by the API Coryne system (bioMérieux) according to the instructions of the manufacturer. For appraisal of the resulting biochemical profile, apiweb identification software was used. The obtained code was 2100124, indicating a good identification at the genus level.

The 16S rRNA gene sequence was amplified as described by Buczolis *et al.* (2008) using the universal primers 27f and 1492r (Lane, 1991) but GoTaq Flexi DNA Polymerase (Promega) was used in PCR. Sequencing was carried out as described by Hilbert *et al.* (2010). The nearly complete sequence (1439 bases) was compared to gene bank entries using the EzTaxon server (Chun *et al.*, 2007) and FASTA (Pearson & Lipman, 1988). The nearest relatives were *Corynebacterium diphtheriae* NCTC 11397^T (96.7 and 96.3 % similarity, respectively), *Corynebacterium pseudotuberculosis* CIP 102968^T (96.6 and 96.3 % similarity, respectively), *Corynebacterium humireducens* MFC-5^T (96.8 and 96.2 %, respectively) and *C. ulcerans* NCTC 7910^T (96.7 and 96.1 % similarity, respectively). These data clearly demonstrate that strain 410^T is a member of the genus *Corynebacterium* but do not suggest affiliation of strain 410^T to any currently recognized species. Multiple alignment of sequences of type strains sharing 95 % or higher similarity in the EzTaxon comparison was performed using CLUSTAL_X (Thompson *et al.*, 1997) and manually edited for ambiguous bases and gaps using BioEdit (Hall, 1999). The phylogenetic position of strain 410^T was examined by the maximum-likelihood, maximum-parsimony and neighbour-joining algorithms implemented in the PHYLIP package (Felsenstein, 2009) applying standard adjustments. Using the maximum-likelihood algorithm, strain 410^T branched at the root of the clade with which the type species of the genus, *C. diphtheriae*, was affiliated (Fig. 1); this position was also supported in the neighbour-joining and maximum-parsimony trees (results

not shown). This branching was not supported by significant bootstrap values (24–54 %). However, phylogenetic analyses confirm assignment of strain 410^T to the genus *Corynebacterium* but separate from any recognized species.

For production of biomass for analyses of mycolic acids, quinones, polar lipids and polyamines, strain 410^T was grown at 28 °C in PYE broth (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) and the reference strains were grown in 3.3 $\times$ PYE broth (0.99 % peptone from casein, 0.99 % yeast extract, pH 7.2). For extraction of mycolic acids, 50 mg lyophilized biomass was suspended in a mixture of 2.5 ml methanol, 2.5 ml toluene and 0.1 ml concentrated sulphuric acid and incubated for 16 h at 75 °C in a tightly closed tube. After cooling down to 4 °C, the mixture was shaken thoroughly with 1 ml hexane. After phase separation, the hexane phase containing mycolic acids was collected and passed through a column containing NH₄HCO₃ to remove sulphuric acid. The hexane phase was taken to dryness under a stream of nitrogen and then dissolved in a final volume of 100 μ l hexane. Approximately 50 μ l of the extract was spotted on a silica gel 60 thin layer plate (200 $\times$ 200 mm, thickness 0.2 mm; Macherey-Nagel) and developed in a mixture of petroleum ether (35–60 °C) : acetone (95:5). Mycolic acids were detected by spraying with 5 % ethanolic molybdatophosphoric acid followed by heating at 160 °C for 15 min. Polar lipids and quinones were extracted and analysed applying the integrated procedure reported by Tindall (1990a, b) and Altenburger *et al.* (1996). Polyamines were extracted as reported by Busse & Auling (1988) and Altenburger *et al.* (1997). For HPLC analyses of quinones and polyamines, the equipment used was as reported by Stolz *et al.* (2007).

Strain 410^T was shown to contain corynemycolic acids that exhibited a slightly higher *R_f* value than those detected in *C. diphtheriae* DSM 20524^T (results not shown).

The quinone system of strain 410^T was composed of MK-8(H₂) (70 %), MK-9(H₂) (26 %) and MK-7(H₂) (4 %), which supports affiliation to the genus *Corynebacterium*.

Strain 410^T exhibited a complex polar lipid profile (Fig. 2). The major compounds were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, four unidentified glycolipids (GL1–GL4) and two unidentified phospholipids (PL1, PL2). Minor amounts of four unidentified polar lipids (L1, L2, L3 and L4) that were not stainable with specific spray reagents such as molybdenum blue, ninhydrin or α -naphthol were also detected. Phosphatidylinositol mannosides, reported to be present in several *Corynebacterium* species (Fudou *et al.*, 2002; Yassin *et al.*, 2003; Chen *et al.*, 2004), and also shown to be present in the reference species examined in the course of this study (Fig. 2), could not be detected. Obviously, this lipid is not generally found in *Corynebacterium* species or it is present in amounts that are below detection limit, as recently also shown for *Corynebacterium lubricantis* (Kämpfer *et al.*, 2009). Strain 410^T showed major differences in its polar lipid profile compared to its close relatives *C. diphtheriae*, *C.*

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the relatedness of strain 410^T to *Corynebacterium* species that were selected based on sequence similarities with strain 410^T higher than 95%. *Mycobacterium tuberculosis* NCTC 7416^T (X58890) was used as an outgroup. Bootstrap values (%) >70% based on 100 replicates are given at nodes. Bar, 0.01 substitutions per nucleotide position.

pseudotuberculosis, *C. ulcerans* and the more distantly related reference species *Corynebacterium glutamicum* (Fig. 2). It did not show the presence of phosphatidylinositol mannoside; the major glycolipids GL2 and GL3 were not detectable in any of the three reference species; glycolipid GL4 was only detected in *C. glutamicum* and glycolipid GL1 was only detected in *C. glutamicum* and *C. diphtheriae*. However, each of the reference species exhibited a distinctive polar lipid profile compared to the other reference species. *C. pseudotuberculosis* and *C. ulcerans*, which are phylogenetically most closely related to each other (Fig. 1), showed most similar lipid profiles, differing only in the presence of the minor lipids PL3 and L6 in *C. ulcerans*. These data indicate that, within the genus *Corynebacterium*, polar lipids reflect close phylogenetic relatedness but also that they have apparently a high discriminative power that may be useful for differentiation of less related species.

The polyamine pattern was composed of spermidine [0.70 µmol (g dry weight)⁻¹], spermine [0.59 µmol (g dry weight)⁻¹], putrescine [0.36 µmol (g dry weight)⁻¹], 1,3-diaminopropane [0.05 µmol (g dry weight)⁻¹] and cadaverine [0.01 µmol (g dry weight)⁻¹]. This polyamine pattern is in line with characteristics reported for several *Corynebacterium* species (Altenburger *et al.*, 1997).

Production of biomass, and extraction and analysis of fatty acids was carried out as described by Kämpfer & Kroppenstedt (1996). The fatty acid profile (Table 1) was composed predominantly of straight-chain, saturated and mono-unsaturated fatty acids and major fatty acids were C_{18:1}ω₉c, C_{16:1}ω₇c and C_{16:0}. Tuberculostearic acid could not be detected. This fatty acid profile was qualitatively quite similar to those of *C. diphtheriae* NCTC 11397^T, *C. pseudotuberculosis* DSM 20689^T and *C. ulcerans* DSM 46325^T.

Fig. 2. Polar lipid profiles of strain 410^T (a), *C. glutamicum* DSM 20300^T (b), *C. diphtheriae* DSM 20524^T (c), *C. pseudotuberculosis* DSM 20689^T (d) and *C. ulcerans* DSM 46325^T (e) after two-dimensional TLC and detection with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; GL1–GL8, unidentified glycolipids; L1–L8, unidentified lipids; PL1–PL3, unidentified phospholipids.

but the presence of major amounts of C_{16:1}ω7c clearly distinguished strain 410^T from *C. glutamicum* DSM 20300^T and *C. humireducens* MFC-5^T (Table 1). However, significant quantitative differences in the contents of several fatty acids, including C_{16:0} and C_{18:1}ω7c, and the presence of C_{17:1}ω8c distinguished strain 410^T from its close relatives.

Results of the comparative physiological characterization using identical test conditions (Kämpfer *et al.*, 1991) for *C. diphtheriae* NCTC 11397^T, *C. pseudotuberculosis* DSM 20689^T, *C. ulcerans* DSM 46325^T and *C. glutamicum* DSM 20300^T are shown in Table 2.

Due to the close relatedness found between strain 410^T and *C. diphtheriae*, production of diphtheria toxin was analysed. The toxigenicity testing was done according to the *Manual for the Laboratory Diagnosis of Diphtheria* by A. Efstratiou and P. A. C. Maple, WHO, Copenhagen 1994 (conventional test) and Engler *et al.* (1997) (modified Elek test). In both the conventional and modified Elek tests, no indication of toxigenicity was detected. These results were also confirmed in a PCR-based assay (results not shown) applying primers for the specific detection of the 'A' portion of the toxin gene (Pallen *et al.*, 1994) and primers for amplification of a 910 bp DNA fragment overlapping

the A and B portions of the genes coding for diphtheria toxin (Hauser *et al.*, 1993).

From the results from 16S rRNA gene analyses, and differences in phenotype (polar lipid and fatty acid profiles and physiology), it is obvious that strain 410^T is different from *C. diphtheriae*, *C. pseudotuberculosis*, *C. ulcerans* and *C. glutamicum* and from other *Corynebacterium* species as well. In conclusion, strain 410^T is a representative of a novel species of the genus *Corynebacterium* for which we propose the name *Corynebacterium epidermidicanis* sp. nov.

Description of *Corynebacterium epidermidicanis* sp. nov.

Corynebacterium epidermidicanis (e.pi.der.mi.di.ca'nis. L. n. *epidermis* -idis the surface, skin; L. n. *canis* -is a dog; N.L. gen. n. *epidermidicanis* of the skin of a dog).

Cells are pleomorphic (coccoid to rods) and non-motile with Gram-positive behaviour. Oxidase-negative. Good growth occurs on PYE agar and blood agar but not on MacConkey agar and it is able to grow under anaerobic conditions. Forms creamy-whitish, non-translucent colonies at 28°C on PYE agar with a diameter of approximately

Table 1. Cellular fatty acid composition of strain 410^T and related strains

Strains: 1, 410^T; 2, *C. diphtheriae* NCTC 11397^T; 3, *C. pseudotuberculosis* DSM 20689^T; 4, *C. ulcerans* DSM 46325^T; 5, *C. glutamicum* DSM 20300^T; 6, *C. humireducens* MFC-5^T [data from Wu *et al.* (2011)]. All data were from this study, except where marked. Data were obtained with the Sherlock MIDI version 2.1 (TSBA version 4.1). Empty cells indicate that the fatty acids were not detected.

Fatty acid	1	2	3	4	5	6
C _{14:0}		1.8	2.9	4.6	0.8	1.6
C _{15:0}	0.7					
iso-C _{15:0}	1.0					
C _{15:1ω6c}	1.0					
C _{16:1ω5c}	3.1					
C _{16:1ω9c}					0.8	
C _{16:1ω7c} *	25.3	36.3	33.6	30.6		
anteiso-C _{17:1ω9c}	1.5					
C _{16:0}	18.0	42.6	51.6	45.2	42.6	33.7
C _{18:1ω9c}	30.6	15.4	9.4	17.6	53.6	42.7
C _{18:1ω7c}	2.6					1.7
C _{17:0}					1.1	3.4
C _{17:1ω8c}	7.9					5.1
C _{17:1ω6c}	3.2					
C _{18:0}	1.3	3.4	2.6	2.3	1.0	7.6

*The MIDI system identified this fatty acid as C_{16:1ω7c} and/or iso-C_{15:0} 2-OH. Since members of the genus *Corynebacterium* are known to lack hydroxy fatty acids, it is most likely that this fatty acid in fact is C_{16:1ω7c}.

0.5 mm. The lipid profile consists of the major compounds diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-inositol, three glycolipids (GL2, GL3 and GL4), moderate amounts of two phospholipids (PL1 and PL2), a glycolipid (GL1) and minor amounts of four polar lipids not stainable with any specific detection reagent (L1, L2, L3 and L4). Major polyamines are spermine and spermidine. The quinone system is composed of predominantly MK-8(H₂) and MK-9(H₂). The fatty acid profile contains large amounts of C_{18:1ω9c}, C_{16:1ω7c} and C_{16:0}. Tuberculostearic acid is absent.

Acetate, propionate, fumarate, L-aspartate, L-histidine and L-serine and trehalose (weakly) are assimilated but *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, *p*-arbutin, cellobiose, gluconate, α-melibiose, L-rhamnose, D-ribose, sucrose, salicin, D-xylose, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, glutarate, DL-3-hydroxybutyrate, itaconate, mesaconate, oxoglutarate, suberate, L-alanine, β-alanine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate are not. In the miniaturized test system reported by Kämpfer *et al.* (1991), acid is not produced from glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol,

Table 2. Physiological test results of strain 410^T and related strains

Strains: 1, 410^T; 2, *C. diphtheriae* NCTC 11397^T; 3, *C. pseudotuberculosis* DSM 20689^T; 4, *C. ulcerans* DSM 46325^T; 5, *C. glutamicum* DSM 20300^T. +, Positive; (+), weakly positive; −, negative.

Test	1	2	3	4	5
pNP-α-D-glucopyranoside hydrolysis	−	(+)	−	−	−
Assimilation of:					
D-Ribose	−	(+)	+	+	+
Trehalose	(+)	−	−	+	−
Acetate	+	(+)	−	+	+
Propionate	+	−	−	+	+
Fumarate	+	−	+	+	+
L-Aspartate	+	(+)	+	+	+
L-Histidine	+	−	−	−	−
L-Serine	+	−	(+)	−	(+)

L-arabinose, raffinose, rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl D-glucoside, erythritol, melibiose, D-arabitol or D-mannitol. Aesculin, *para*-nitrophenyl (pNP)-β-D-galactopyranoside, pNP-β-D-glucuronide, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, pNP-β-D-xylopyranoside, bis-pNP-phosphate, pNP-phenyl-phosphonate, pNP-phosphoryl-choline, 2-deoxythymidine-5'-pNP-phosphate and L-glutamate-γ-3-carboxy-*p*-nitroanilide are not hydrolysed.

In the ApiCoryne test, produces acid from the carbohydrates D-glucose and maltose, whereas no acid is produced from D-ribose, D-xylose, D-mannitol, lactose, sucrose or glycogen. Alkaline phosphatase and pyrazinamidase are positive, whereas β-glucuronidase, β-galactosidase, α-glucosidase, *N*-acetylglucosaminidase and urease production are negative. Nitrate reduction and aesculin and gelatin hydrolysis are negative; catalase is positive. Non-lipophilic and the CAMP reaction is positive. The resulting ApiCoryne code is 2100124. Diphtheria toxin is absent.

The type strain is 410^T (=DSM 45586^T=LMG 26322^T=CCUG 60915^T), isolated in Austria from the skin of a dog suffering from pruritus.

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