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Corynebacterium freiburgense sp. nov., isolated from a wound obtained from a dog bite

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A non-lipophilic, coryneform bacterium, isolated from a patient's wound obtained from a dog bite, was characterized by phenotypic, chemotaxonomic and molecular genetic methods. Chemotaxonomic features suggested assignment of the unknown bacterium to the genus *Corynebacterium*. The isolate exhibited the following peculiar features which made it possible to differentiate it phenotypically from all other medically relevant corynebacteria: older colonies exhibited a 'spoke-wheel' macroscopic morphology, colonies were strongly adherent to blood agar and the strain did not have pyrazinamidase activity, but was positive for β-galactosidase. 16S rRNA gene sequencing showed that the closest phylogenetic relative exhibited more than 3.9 % divergence from the unknown isolate. Based on phenotypic and molecular genetic data, it is proposed that the isolate should be classified as a representative of a novel species, *Corynebacterium freiburgense* sp. nov., with strain 1045^T (=CCUG 56874^T =DSM 45254^T) as the type strain.

During the 1990s, a plethora of novel *Corynebacterium* species isolated from human clinical specimens was described (Funke & Bernard, 2007). Within the last few years, microbiologists have also focused on descriptions of novel *Corynebacterium* species obtained from animals (Collins *et al.*, 1999b, 2001, 2004; Fernández-Garayzábal *et al.*, 2004). Although it is generally agreed that the most frequently found *Corynebacterium* species in human clinical materials have already been defined, novel *Corynebacterium* species are still being described, often based, however, on single strains (Yassin *et al.*, 2002; Yassin, 2007). The present report outlines the characteristics of a single, unusual *Corynebacterium* strain (1045^T) which may have been transmitted from an animal to a human. Using a polyphasic taxonomic approach, it has been demonstrated that this strain represents another novel *Corynebacterium* species.

Strain 1045^T was cultured in August 2008 from a wound swab of a 57-year-old female who had been bitten by her dog on her forearm. Strain 1045^T grew together with *Pasteurella multocida*, α-haemolytic streptococci and *Prevotella* species.

Gram staining of cells of strain 1045^T showed coryneform bacteria arranged singly with typical club-shaped elements; filamentous forms were not observed. The isolate was negative for partial acid-fastness. Colonies on Columbia sheep blood agar plates (BD) were beige–whitish, showed irregular margins and were 1–2 mm in diameter. Supplementation of Columbia sheep blood agar plates with Tween 80 (Merck) (Funke & Bernard, 2007) did not enhance colony size significantly, i.e. strain 1045^T was non-lipophilic. Interestingly, the colonies were strongly adherent to blood agar; of the coryneform bacteria, adherence to agar is also observed in some strains of *Corynebacterium durum* (Riegel *et al.*, 1997), *Corynebacterium sundsvallense* (Collins *et al.*, 1999a) and *Corynebacterium thomssenii* (Zimmermann *et al.*, 1998). Another very peculiar feature was the 'spoke-wheel' macroscopic morphology of the colonies after 5 days of incubation at 37 °C in a 5 % CO_2-enriched atmosphere. This type of morphology is not seen in other true corynebacteria, but may be observed in some *Rothia dentocariosa* strains (Funke & Bernard, 2007).

Strain 1045^T was further screened for chemotaxonomic features and biochemical reactions using previously described methods (Funke *et al.*, 1993). Chemotaxonomic investigations revealed the presence of *meso*-diaminopimelic acid as diamino acid of the peptidoglycan, as well as mycolic acids, features which, together with the negative reaction for partial acid-fastness, are compatible with the assignment of strain 1045^T to the genus

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 1045^T is FJ157329.

Corynebacterium (Funke & Bernard, 2007). The main straight-chain saturated fatty acids were palmitic and stearic acids; oleic acid was the predominant unsaturated fatty acid.

When applying the commercial API Coryne (bioMérieux), a negative pyrazinamidase reaction was observed (numerical API Coryne code: 1440365), which prompted us to consider that the isolate belonged to the *Corynebacterium diphtheriae*/*C. ulcerans*/*C. pseudotuberculosis* group of bacteria, since these bacteria are the only known large-colony-forming, medically relevant corynebacteria that do not express this particular enzyme continuously (Funke & Bernard, 2007). As a result of this and because of the clinical nature of the patient's wound, the isolate was tested for the presence of the diphtheria toxin gene using PCR primers Cdipht-1 (5′-ATCCACTTTTAGTGCGAGAACC TTGGTCA) and Cdipht-2 (5′-GAAAACTTTTCTTCG TACCACGGGACTAA), as outlined previously (Nakao & Popovic, 1997). Results showed that strain 1045^T did not harbour this virulence gene.

Another unusual feature for a medically relevant corynebacterium was the positive β-galactosidase reaction of strain 1045^T in both the API Coryne (at pH 7.4) and the API ZYM (at pH 5.4) (bioMérieux) systems. *C. durum* (Rassoulian Barrett *et al.*, 2001) and *Corynebacterium glucuronolyticum* (Funke *et al.*, 1995) are the only other clinically significant true corynebacteria that express this enzyme. Two further features of strain 1045^T are also not observed frequently in other clinical corynebacteria (Funke & Bernard, 2007), i.e. a positive aesculinase reaction (delayed, turning positive after 72 h incubation only) and the ability to ferment lactose [tested with both the API Coryne and the API 50CH (bioMérieux) systems].

In summary, strain 1045^T exhibited some very unusual phenotypic features that are not compatible with any *Corynebacterium* species with validly published names. Therefore, the phylogenetic distinctiveness of strain 1045^T was investigated by sequencing the almost-entire 16S rRNA gene (1481 bp) according to a published method (Beck *et al.*, 2008). Strain 1045^T clustered with the type strains of the 70 currently recognized species of the genus *Corynebacterium* and 16S rRNA gene sequence similarities ranged from 91.54 % with *C. durum* to 96.06 % with *C. pseudotuberculosis*. The ten closest phylogenetic relatives of the isolate were the type strains of *C. pseudotuberculosis* (96.06 % 16S rRNA gene sequence similarity), *Corynebacterium vitaeruminis* (95.92 %), *C. ulcerans* (95.82 %), *Corynebacterium felinum* (95.81 %), *Corynebacterium spheniscorum* (95.60 %), *Corynebacterium argentoratense* (95.57 %), *Corynebacterium variabile* (95.57 %), *Corynebacterium aquilae* (95.55 %), *C. diphtheriae* (95.34 %) and *Corynebacterium falsenii* (95.19 %). As expected, the type strains of members of the genera *Dietzia*, *Rhodococcus* and *Tsukamurella* were also related phylogenetically to the isolate, with 16S rRNA gene sequence similarities of approximately 92 %. It is evident from the molecular genetic data, which clearly shows 16S rRNA gene sequence divergence above the 3 % threshold (Stackebrandt & Goebel, 1994), that strain 1045^T represents a novel *Corynebacterium* species. Table 1 outlines phenotypic features that enable the isolate to be differentiated clearly from its nearest phylogenetic relatives. A phylogenetic tree was constructed using the neighbour-joining method included in the MEGA4 software suite (Tamura *et al.*, 2007), based on a comparison of approximately 1350 nt. Bootstrap values, expressed as percentages of 1000 replications, are given at each branching point in Fig. 1. From the treeing analysis, it is evident that strain 1045^T represents a distinct *Corynebacterium* species.

Antimicrobial susceptibility testing was performed using the microdilution method of the Clinical and Laboratory Standards Institute (CLSI), as well as the interpretation guidelines of this organization (CLSI, 2006). Strain 1045^T was susceptible to cefotaxime, ciprofloxacin, doxycycline, erythromycin, gentamicin, linezolid, meropenem, penicillin, rifampicin and vancomycin. The strain was also susceptible to the vibriocidal compound O/129 (Funke *et al.*, 1996).

It is interesting to note that, among the *Corynebacterium* species, only a few species, e.g. *Corynebacterium auriscanis* (Collins *et al.*, 1999b) and *C. ulcerans* (Lartigue *et al.*, 2005), have so far been isolated from dogs. However, in our experience, it is not unlikely that many other *Corynebacterium* species (and even some novel species) might be detected in clinical materials from dogs if samples are screened more systematically for the presence of corynebacteria.

Based on the results of the outlined polyphasic taxonomic study, it is proposed that strain 1045^T represents a novel species of the genus *Corynebacterium*, *Corynebacterium freiburgense* sp. nov.

Table 1. Characteristics that differentiate strain 1045^T from its nearest phylogenetic relatives

Species: 1, *C. freiburgense* sp. nov. (strain 1045^T); 2, *C. felinum*; 3, *C. pseudotuberculosis*; 4, *C. ulcerans*; 5, *C. vitaeruminis*. +, Positive; −, negative; V, variable; ND, no data. Data from this study, Collins *et al.* (2001) and Funke & Bernard (2007).

Characteristic	1	2	3	4	5
Pyrazinamidase	−	+	−	−	+
Nitrate reductase	+	−	−	−	+
Urease	−	−	+	+	+
Pyrrolidonyl arylamidase	−	+	−	−	+
α-Glucosidase	−	+	V	+	ND
β-Glucosidase	+	−	−	−	+
β-Galactosidase	+	−	−	−	ND
Acid production from lactose	+	−	−	−	−

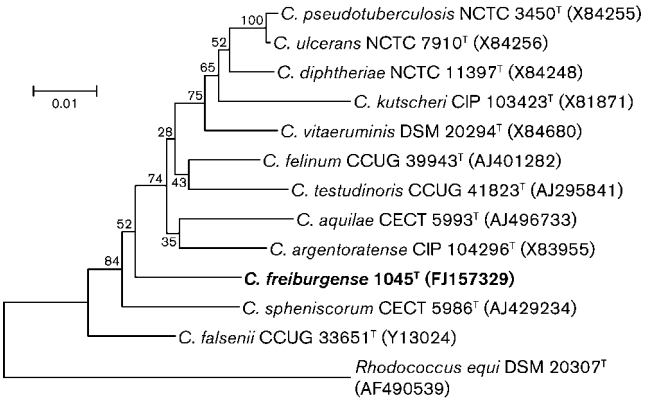

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain 1045^T in relation to the type strains of its closest phylogenetic neighbours; corresponding GenBank sequence accession numbers are given. The sequence of *Rhodococcus equi* DSM 20307^T was used as an outgroup. Bar, 0.01 nucleotide substitutions per site. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points.

Description of *Corynebacterium freiburgense* sp. nov.

Corynebacterium freiburgense (frei.bur.gen′se. N.L. neut. adj. *freiburgense* from Freiburg/Breisgau, Germany, named after the city where the bacterium was first isolated).

Cells stain Gram-positive and are non-spore-forming and non-motile. They are typically club-shaped rods that occur as single cells, in pairs or in small clusters. Colonies are beige–whitish, dryish, convoluted with irregular edges, strongly adherent to sheep blood agar plates and up to 1–2 mm in diameter after 48 h incubation. In 5-day-old colonies, a 'spoke-wheel' macroscopic morphology may be observed. Weak anaerobic growth is observed. Catalase-positive. Acid is produced from fructose, galactose, glucose, 5-ketogluconate, lactose, maltose, mannose, ribose, sucrose and tagatose, but not from *N*-acetylglucosamine, adonitol, amygdalin, arabinose, arabitol, arbutin, cellobiose, dulcitol, erythritol, fucose, gentiobiose, gluconate, glycerol, inositol, inulin, 2-ketogluconate, lyxose, mannitol, melezitose, melibiose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-xyloside, raffinose, rhamnose, salicin, sorbitol, sorbose, starch, trehalose, turanose, xylitol or xylose. The following enzyme activities can be detected: nitrate reductase, β-galactosidase, β-glucosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, acid phosphatase and phosphoamidase. Pyrazinamidase, urease, pyrrolidonyl arylamidase, gelatinase, lipase, valine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are not detected. The CAMP (Christie–Atkins–Munch-Petersen) reaction is negative. The cell wall contains *meso*-diaminopimelic acid and mycolic acids are also present. The main straight-chain saturated fatty acids are palmitic and stearic acids; oleic acid is the predominant unsaturated fatty acid.

The type strain is 1045^T (=CCUG 56874^T =DSM 45254^T), isolated from a patient's wound obtained from a dog bite.

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