Corynebacterium pilbarensense sp. nov., a non-lipophilic corynebacterium isolated from a human ankle aspirate

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A non-lipophilic coryneform bacterium isolated from an anaerobic Bactec bottle inoculated with an ankle aspirate from a male patient was characterized by phenotypic and molecular taxonomic methods. Chemotaxonomic investigations revealed the presence of short-chain mycolic acids in the cell wall of the bacterium, a feature consistent with members of the genus *Corynebacterium*. Comparative 16S rRNA gene sequence analysis demonstrated that the isolate displayed 92.0–99.0 % gene sequence similarity with members of the genus *Corynebacterium*, with *Corynebacterium ureicelerivorans* as the most closely related phylogenetic species (99.0 % gene sequence similarity). However, the isolate could be genomically separated from *C. ureicelerivorans* on the basis of DNA–DNA hybridization studies (39.5 % relatedness). Furthermore, the isolate could also be differentiated from *C. ureicelerivorans* and other species of the genus *Corynebacterium* on the basis of biochemical properties. Based on both phenotypic and phylogenetic evidence, it is proposed that this isolate be classified as representing a novel species, *Corynebacterium pilbarensense* sp. nov. (type strain IMMIB WACC 658^T=DSM 45350^T=CCUG 57942^T).

The genus *Corynebacterium* is one of the largest genera within the class *Actinobacteria* and currently embraces over 82 species, a high proportion of which have been defined over the past decade. Some corynebacteria are well-established pathogens of man and animals, whereas many others occur as part of the normal flora of skin and mucous membranes (Soriano and Fernandez-Roblas, 1988; Coyle & Lipsky, 1990; Colt *et al.*, 1991; Sewell *et al.*, 1995). As some of these diphtheroids show a lipid requirement for growth, which can be supplied by serum or Tween 80, these strains have often been referred to as lipophilic diphtheroids. Attempts to classify these organisms on the basis of biochemical characteristics have been undertaken. The Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) separated lipophilic diphtheroids into groups ANF-1, G-1 and G-2 in addition to groups JK (*Corynebacterium jeikeium*) and D-2 (*Corynebacterium urealyticum*) using biochemical tests (Riley *et al.*, 1979; Hollis & Weaver, 1981; Riegel *et al.*, 1992, 1993). After several different taxonomic studies, it was concluded that

the strains formerly named as CDC group G-1 represent at least two species, *Corynebacterium macginleyi* (Riegel *et al.*, 1995) and *Corynebacterium accolens* (Neubauer *et al.*, 1991). Other glucose-fermenting lipid-requiring strains that do not hydrolyse urea are now known as *Corynebacterium tuberculostearicum* (Brown *et al.*, 1984; Feurer *et al.*, 2004). During the course of characterization of bacterial isolates encountered from clinical sources, we found a Gram-positive, pleomorphic to rod-shaped, glucose-fermenting bacterium that did not hydrolyse urea. The API Coryne profile assigned the organism to CDC group G. Further chemotaxonomic and phylogenetic investigations confirmed its assignment to the genus *Corynebacterium* and indicated that it was different from previously described species of the genus. Based on both phenotypic and phylogenetic evidence, we propose that this strain represents a novel species of the genus *Corynebacterium*.

Strain IMMIB WACC 658^T was isolated from anaerobic Bactec bottles inoculated with ankle aspirate from a 54-year-old man, probably with gout (urate crystals present in the joint fluid) in Pilbara, West Australia. The strain was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB WACC 658^T is FN295567.

submitted to the Reference Laboratory of the Institute of Medical Microbiology, University of Bonn, Germany, for identification. The isolate was cultured on Columbia blood agar supplemented with 5% sheep blood to determine its morphological properties. Lipophilic requirement was determined according to standard procedures (Riegel *et al.*, 1994). Fermentation and enzymic tests were performed using the API Coryne, API 20 Strep and the API ZYM systems according to the manufacturer's instructions (bioMérieux), except for the time of incubation. Enzyme reactions and acid production from carbohydrates, using the API Coryne and API Strep systems, were read after 48 h incubation at 37 °C. The isomeric form of diaminopimelic acid was determined by the methods of Becker *et al.* (1964) and whole-cell sugars were determined according to the method of Lechevalier (1968). Lipids were extracted using acid methanolysis and mycolic acids were detected with TLC as described by Minnikin *et al.* (1980). A standard procedure was used to determine the fatty acid profile (Minnikin *et al.*, 1980; Yassin *et al.*, 2007).

For phylogenetic analysis, 16S rRNA genes were amplified by PCR using procedures described previously (Rainey *et al.*, 1996) and directly sequenced using a Taq dye-deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 310; Applied Biosystems). The closest relatives of the isolate were determined by performing database searches. Phylogenetic analysis was carried out as previously described (Yassin, 2009) using the ARB-package (Ludwig *et al.*, 2004). DNA–DNA relatedness studies were performed between strain IMMIB WACC 658^T and *C. ureicelerivorans* DSM 45051^T. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

Strain IMMIB WACC 658^T was Gram-positive, non-motile, non-spore-forming and had pleomorphic to short rod-shaped cells. On Columbia blood agar, circular, large colonies (approx. 0.5–2.0 mm diameter) were formed after 24 h incubation at 37 °C. The colonies were non-haemolytic. The organism was facultatively anaerobic and was catalase-positive, but oxidase- and urease-negative. The API Coryne numerical profile was 6100305, which corresponded to the numerical profile for *Corynebacterium* group G (ID 96.7%). However, the main difference between strain IMMIB WACC 658^T and *Corynebacterium* group G was that the latter includes lipid-requiring strains, whereas strain IMMIB WACC 658^T was non-lipophilic. An examination of cell-wall murein acid hydrolysates of the novel strain revealed the presence of *meso*-diaminopimelic acid as the dibasic amino acid. TLC analysis of cell-wall sugars revealed the presence of galactose and arabinose, i.e.

the organism had cell-wall chemotype IV *sensu* Lechevalier & Lechevalier (1970). Lipid analysis revealed the presence of corynemycolic acids. Examination of the non-hydroxylated long-chain cellular fatty acids of the novel strain showed the presence of straight-chain saturated C_{16:0} (31.6%), C_{17:0} (0.5%), C_{18:0} (10.9%), monounsaturated C_{16:1}ω7c (0.4%), C_{17:1}ω9c (0.1%), C_{18:1}ω9c (56.0%) and *cis*-9,12-octadecadienoic acid C_{18:2} (0.4%). Tuberculostearic acid was not detected. These chemotaxonomic characteristics, together with the morphological and biochemical properties of isolate IMMIB WACC 658^T, were strongly indicative that the organism belonged to the genus *Corynebacterium*.

To establish the phylogenetic position of strain IMMIB WACC 658^T, its 16S rRNA gene sequence (1486 nt) was determined in this study. Sequence database searches revealed that strain IMMIB WACC 658^T was most closely related to species of the genus *Corynebacterium* (data not shown). Phylogenetic analysis confirmed the placement of strain IMMIB WACC 658^T within the genus *Corynebacterium*. A tree constructed using the neighbour-joining method showing the phylogenetic position of strain IMMIB WACC 658^T in relation to members of the genus *Corynebacterium* is presented in Fig. 1. Comparative 16S rRNA gene sequence analysis unequivocally demonstrated that the isolate represented a hitherto unknown species of the genus *Corynebacterium* that formed a distinct subline within a subcluster of species including the two subspecies of *Corynebacterium afermentans*, *Corynebacterium coyleae*, *Corynebacterium mucifaciens* and *C. ureicelerivorans*. Highest sequence similarity values were shown with *C. ureicelerivorans* (99.0% similarity), the two subspecies of *C. afermentans* (98.9%), *C. coyleae* (98.7%), and *C. mucifaciens* (98.2%). Although the new strain showed close affinity with the aforementioned species, bootstrap resampling analysis showed that this association was not particularly significant. Significantly lower levels of sequence similarity were shown to other members of the genus *Corynebacterium* (data not shown).

Despite the high sequence similarity between isolate IMMIB WACC 658^T and *C. ureicelerivorans*, the novel strain represented a distinct genomic species. It is now recognized that a 16S rRNA gene sequence similarity range above 98.7–99% should be mandatory for establishing the genomic uniqueness of a novel isolate (Stackebrandt & Ebers, 2006). In addition, the DNA–DNA relatedness study between strain IMMIB WACC 658^T and *C. ureicelerivorans* DSM 45051^T clearly demonstrated that the novel strain belonged to a separate genomic species. The mean DNA–DNA relatedness value was 39.5%, a value well below the 70% cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987). Strain IMMIB WACC 658^T could also be distinguished from *C. ureicelerivorans* using a combination of phenotypic properties (Table 1). In addition, strain IMMIB WACC 658^T could be differentiated from the frequently encountered species *Corynebacterium amycolatum* and

Fig. 1. Neighbour-joining phylogenetic tree showing the position of strain IMMIB WACC 658^T within the radiation of species of the genus *Corynebacterium*. Numbers at nodes indicate the level of bootstrap support (%) based on neighbour-joining analyses of 500 resampled datasets; solid circles indicate that the corresponding nodes (groupings) were also recovered in the maximum-likelihood and maximum-parsimony trees. Bar, 5.0% sequence divergence.

Corynebacterium minutissimum on the basis of the presence of mycolic acids and absence of tuberculostearic acid, respectively. The novel strain could also be differentiated from *Corynebacterium striatum* and the less frequently seen *Corynebacterium simulans* on the basis of nitrate reduction.

Thus the 1.0% 16S rRNA gene sequence divergence between strain IMMIB WACC 658^T and its closest phylogenetic neighbour *C. ureicelerivorans*, together with the distinctive phenotype of strain IMMIB WACC 658^T (Table 1), clearly demonstrated that this new isolate represents a novel species. Hence, on the basis of both phenotypic and phylogenetic evidence, the new strain merits classification as a novel species of the genus

Corynebacterium, for which the name *Corynebacterium pilbarensis* sp. nov. is proposed.

Description of *Corynebacterium pilbarensis* sp. nov.

Corynebacterium pilbarensis (pil.ba.ren'se. N.L. neut. adj. *pilbarensis* pertaining to Pilbara, West Australia, the region from which the strain was isolated).

Cells are Gram-positive, non-spore-forming, non-motile, pleomorphic to short rods. Colonies are creamy, circular and approximately 0.5–2.0 mm in diameter on Columbia blood agar after 24 h incubation at 37 °C. Colonies are non-haemolytic. Facultatively anaerobic, catalase-positive and oxidase-negative. Non-lipophilic. Aesculin, gelatin and hippurate are not hydrolysed. Urease and nitrate reduction are negative. Acid is produced from D-glucose, D-ribose and sucrose. Acid is not produced from L-arabinose, glycogen, inulin, lactose, maltose, mannitol, raffinose, sorbitol, trehalose or D-xylose. Activity for alkaline and acid phosphatases, leucine arylamidase, pyrazinamidase, pyrrolidonyl arylamidase and naphthol-AS-BI-phosphohydrolase is detected. No activity is detected for arginine dihydrolase, esterase C4, ester lipase C8, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, chymotrypsin, trypsin, valine arylamidase or cystine arylamidase. Acetoin production is negative. Mycolic acids are present. Long-chain fatty acids are of the straight-chain saturated and monounsaturated types, with C_{16:0} and C_{18:1ω9c} predominating; tuberculostearic acid is absent.

The type strain, IMMIB WACC 658^T (=DSM 45350^T =CCUG 57942^T), was isolated from an anaerobic Bactec vial inoculated with an ankle aspirate from a man who was thought to be suffering from gout.

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Table 1. Characteristics that differentiate strain IMMIB WACC 658^T from its closest phylogenetic relatives

Strains/species: 1, IMMIB WACC 658^T (data from this study); 2, *C. ureicelerivorans* IMMIB RIV-2301^T (data from this study); 3, *C. mucifaciens* (8 strains, Funke *et al.*, 1997a); 4, *C. afermentans* subsp. *afermentans* CIP 103499^T (Riegel *et al.*, 1993); 5, *C. afermentans* subsp. *lipophilum* CIP 103500^T (Riegel *et al.*, 1993); 6, *C. coyleae* CCUG 35014^T (Funke *et al.*, 1997b); 7, *C. macginleyi* (Riegel *et al.*, 1995); 8, *C. accolens* CIP 104783^T (Neubauer *et al.*, 1991); 9, *C. tuberculostearicum* (11 strains, Feurer *et al.*, 2004). +, Positive; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Lipid requirement	–	+	–	–	+	–	+	+	+
Nitrate reduction	–	–	–	–	–	–	+	+	v
Urease	–	+	–	–	–	–	–	–	–
Pyrazinamidase	+	+	+	+	+	+	–	+	+
Alkaline phosphatase	+	+	+	+	+	+	+	–	+
Acid production from									
Glucose	+	+	+	–	–	+	+	+	+
Maltose	–	–	–	–	–	–	–	+	v
Sucrose	+	–	v	–	–	–	+	v	v
Tuberculostearic acid	–	+	+	–	–	–	ND	ND	+

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