Corynebacterium sputi sp. nov., isolated from the sputum of a patient with pneumonia

A. F. Yassin¹ and C. Siering²

Correspondence

A. F. Yassin

yassin@mibi03.meb.uni-bonn.de

¹Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, Sigmund-Freud-Straße 25, D-53127 Bonn, Germany

²Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, D-53121 Bonn, Germany

A coryneform bacterium isolated from the sputum of a patient with pneumonia was characterized by phenotypic and molecular taxonomic methods. Chemotaxonomic investigations revealed the presence of cell-wall chemotype IV and short chain mycolic acids consistent with the genus *Corynebacterium*. Comparative 16S rRNA gene sequencing studies confirmed this assignment, with the organism forming a hitherto unknown subline within the genus associated with a subcluster containing *Corynebacterium hansenii*, *Corynebacterium frenetii*, *Corynebacterium xerosis*, *Corynebacterium amycolatum* and *Corynebacterium sphenisci*. Sequence divergence values of >2.7% from established corynebacterial species suggested that the new isolate represented a novel species. This was also supported by the results of the biochemical tests. On the basis of phenotypic and phylogenetic evidence, it is proposed that the unknown bacterium be classified as a novel species of the genus *Corynebacterium*, *Corynebacterium sputi* sp. nov. (type strain IMMIB L-999^T=DSM 45148^T=CCUG 55795^T).

Currently the genus *Corynebacterium* comprises more than 80 species, a high proportion of which have been described relatively recently as a result of the use of much-improved phenotypic and molecular identification methods. Most of the newly described species of the genus *Corynebacterium* have been isolated from human (e.g. Funke *et al.*, 1997a, 1998; Renaud *et al.*, 2001, 2007; Yassin *et al.*, 2002a, b) or animal (e.g. Fernández-Garayzábal *et al.*, 1998, 2004; Goyache *et al.*, 2003; Collins *et al.*, 2001, 2004) clinical sources. During the course of the characterization of bacterial isolates encountered from clinical sources, we found a Gram-positive, rod-shaped organism which had chemotaxonomic characteristics consistent with its provisional assignment to the genus *Corynebacterium*. Further taxonomic and phylogenetic investigations indicated that the novel strain was different from previously described species of the genus *Corynebacterium*. Based on phylogenetic and phenotypic evidence, it is proposed that this isolate, designated IMMIB L-999^T, is a novel species of the genus *Corynebacterium*.

Strain IMMIB L-999^T was isolated from the sputum of a patient with pneumonia. *Corynebacterium hansenii* DSM

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB L-999^T (=DSM 45148^T=CCUG 55795^T) is AM930556 and that for *Corynebacterium hansenii* DSM 45109^T is AM946639.

An extended version of the neighbour-joining phylogenetic tree is available with the online version of this paper.

45109^T was obtained from the German Culture collection (DSMZ) and used as a reference strain. The novel strain was isolated on Columbia agar (Oxoid) supplemented with 5% sheep blood after incubation at 37 °C for 48 h under aerobic conditions. It was characterized biochemically using the API Coryne, API 20 Strep and API ZYM systems according to the manufacturer's instructions (bioMérieux), except for the time of incubation. Lipid requirements were determined according to a standard procedure (Riegel *et al.*, 1994). The isomeric form of diaminopimelic acid in the cell-wall peptidoglycan was determined according to the methods of Becker *et al.* (1964) and whole-cell sugars were determined by 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). Fatty acids were analysed as described recently by Yassin *et al.* (2007).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA and the purification of PCR products were carried out using previously described procedures (Rainey *et al.*, 1996). Purified PCR products were sequenced using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) as described in the manufacturer's protocol. An Applied Biosystems 310 DNA Genetic Analyzer was used for the electrophoresis of the sequence reaction products. The 16S rRNA gene sequence of strain IMMIB L-999^T as well as those of the recognized species of the genus *Corynebacterium* retrieved from the GenBank were

added to the ARB-database (Ludwig *et al.*, 2004) and aligned using the appropriate tool from the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. The evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). The topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

Cells of strain IMMIB L-999^T stained Gram-positive and on microscopic examination appeared as irregular short,

non-motile, non-spore-forming rods. Colonies were cream-yellow, circular, convex, dry and approximately 0.2–2 mm in diameter on Columbia blood agar after 48 h incubation at 37 °C. Colonies were non-haemolytic. The novel organism was facultatively anaerobic, catalase-positive and oxidase-negative. Using the API Coryne test V2.1, positive results were obtained for acid production from glucose and enzymic activity was detected for α -glucosidase, pyrazinamidase and urease. The API Coryne code for strain IMMIB L-999^T was 2011104. Using the API ZYM test, activity was detected for ester lipase C8, ester lipase C14, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. All other enzyme tests were

Fig. 1. Neighbour-joining phylogenetic tree showing the position of strain IMMIB L-999^T within the radiation of species of the genus *Corynebacterium*. The tree was based on a comparison of 16S rRNA gene sequences that were at least 90% complete (with regard to the sequence of *Escherichia coli*). The numbers at the nodes indicate the level of bootstrap support (%) based on neighbour-joining analyses of 1000 resampled datasets; solid circles indicate that the corresponding nodes (groupings) are also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 5.0% sequence divergence. The full tree from which this figure was taken is available as Supplementary Fig. S1 in IJSEM Online.

negative using these kits. An examination of cell-wall murein acid hydrolysates of strain IMMIB L-999^T revealed the presence of *meso*-diaminopimelic acid as the diagnostic diamino acid. TLC analysis of cell-wall sugars revealed the presence of galactose and arabinose, i.e. the organism possessed 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 fatty acids C_{16:0} (20.96 % of total fatty acids), C_{17:0} (0.61 %), C_{18:0} (17.98 %), monounsaturated fatty acids C_{18:1 ω 9c} (33.68 %), C_{19:1 ω 9c} (2.04 %) and C_{20:1} (0.38 %), diunsaturated fatty acid C_{18:2 ω 6,9c} (0.67 %) and tuberculostearic acid 10 Me-C_{18:0} (20.48 %). These chemotaxonomic characteristics, together with the morphological and biochemical properties, were strongly indicative that the novel organism belonged to the genus *Corynebacterium*.

To establish the phylogenetic position of strain IMMIB L-999^T, the 16S rRNA gene sequence was determined in this study (1495 bp). Sequence database searches revealed that the novel bacterium was most closely related to species of the genus *Corynebacterium* (data not shown). Treeing analysis confirmed the placement of strain IMMIB L-999^T within the genus *Corynebacterium*. A tree constructed using the neighbour-joining method showing the phylogenetic position of strain IMMIB L-999^T in relation to recognized 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*. Strain IMMIB L-999^T displayed relatively high 16S rRNA gene sequence similarity values (divergence values greater than 2.4 %) from other recognized species within the genus *Corynebacterium*. Highest sequence similarity values were shown with *C. hansenii* DSM 45109^T (97.3 % similarity), *Corynebacterium xerosis* DSM 20743^T (97.1 %), *Corynebacterium freneyi* CCUG 45704^T (96.4 %), *Corynebacterium sphenisci* CECT 5990^T (96.0 %) and *Corynebacterium amycolatum* NCFB 2768^T (95.8 %). Significantly lower levels of relatedness were shown to other members of the genus *Corynebacterium* (data not shown). Although the novel bacterium showed close affinity with the aforementioned species, bootstrap resampling analysis showed that this association was not particularly significant.

The closest genetic relative to isolate IMMIB L-999^T was *C. hansenii* DSM 45109^T displaying high 16S rRNA gene sequence similarity (2.7 % divergence). Despite the relatively high sequence similarity between isolate IMMIB L-999^T and this species, isolate IMMIB L-999^T 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, it is important to note that many genomically distinct species within the genus *Corynebacterium*, including *Corynebacterium pseudodiphtheriticum* and *Corynebacterium propinquum*

(>99 % 16S rRNA gene sequence similarity), *Corynebacterium mucifaciens* and *Corynebacterium afermentans* (98.5 % similarity) and *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* (>98 % similarity) (Pascual *et al.*, 1995; Ruimy *et al.*, 1995; Funke *et al.*, 1997b), exhibit comparable, or even higher, levels of sequence similarity. Phenotypically, isolate IMMIB L-999^T could be differentiated from *C. hansenii* by its ability to produce urease and its inability to produce alkaline phosphatase and acids from maltose, sucrose and ribose. Therefore, on the basis of both phenotypic and molecular genetic evidence, we are of the opinion that isolate IMMIB L-999^T merits classification as a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium sputi* sp. nov. is proposed. Tests that serve to differentiate *Corynebacterium sputi* sp. nov. from related members of the genus *Corynebacterium* are shown in Table 1.

Description of *Corynebacterium sputi* sp. nov.

Corynebacterium sputi sp. nov. (spu'ti. L. gen. n. *sputi* of sputum).

Cells are Gram-positive, non-spore-forming, non-motile rods. Colonies are cream-yellow, circular, convex, dry and approximately 0.2–2 mm in diameter on Columbia blood agar after 48 h incubation at 37 °C. Colonies are non-haemolytic. Tween 80 encourages growth of the organism. Facultatively anaerobic, catalase-positive and oxidase-negative. Urea is hydrolysed, but aesculin, gelatin and hippurate are not hydrolysed. Nitrate is not reduced. Acid is produced from D-glucose, but not from L-arabinose, glycogen, inulin, D-lactose, maltose, D-mannitol, raffinose, D-ribose, D-sorbitol, starch, sucrose, trehalose, or D-xylose. Activity is detected for α -glucosidase, lipase C8, lipase C14, leucine arylamidase, pyrazinamidase and naphthol-AS-BI-phosphohydrolase. No activity is detected for acid and

Table 1. Characteristics that differentiate strain IMMIB L-999^T from its closest phylogenetic relatives

Strains: 1, strain IMMIB L-999^T (data from this study); 2, *C. hansenii* DSM 45109^T (this study); 3, *C. xerosis* (Funke *et al.*, 1997b; Goyache *et al.*, 2003); 4, *C. freneyi* (Renaud *et al.*, 2001); 5, *C. sphenisci* CCUG 46398^T (Goyache *et al.*, 2003); 6, *C. amycolatum* (Collins *et al.*, 1988; Funke *et al.*, 1997b). +, Positive; –, negative; v, variable; w, weakly positive.

Characteristic	1	2	3	4	5	6
Lipid requirement	+	+	–	–	–	–
Nitrate reduction	–	–	v	v	+	v
Production of:						
Alkaline phosphatase	–	+	+	+	+	+
Urease	+	–	–	–	–	v
Acid production from:						
Maltose	–	+	+	+	+	v
Ribose	–	+	+	w	–	+
Sucrose	–	+	+	+	–	v

alkaline phosphatases, arginine dihydrolase, chymotrypsin, cysteine arylamidase, esterase C-4, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, pyrrolidonyl arylamidase, trypsin or valine arylamidase. Acetoin production is negative. Corynemycolic acids are present. The long-chain cellular fatty acids are of the straight-chain saturated and monounsaturated types, with C_{16:0}, C_{18:0} and C_{18:1 ω 9c} predominating; tuberculostearic acid is present.

The type strain, IMMIB L-999^T (=DSM 45148^T=CCUG 55795^T), was isolated from the sputum of a patient with pneumonia.

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