NOTE

***Corynebacterium appendicis* sp. nov.**A. F. Yassin,¹ U. Steiner² and W. Ludwig³

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A lipophilic, coryneform bacterium isolated from a human clinical specimen 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*. The isolate could be distinguished from other members of the genus *Corynebacterium* by positive urease and catalase tests as well as its failure to produce acid from carbohydrates. Comparative 16S rRNA gene sequencing showed that this isolate constitutes a distinct subline within the genus *Corynebacterium*, displaying >3.0% sequence divergence from other known *Corynebacterium* species. Based on both phenotypic and phylogenetic evidence, it is proposed that this isolate be classified as a novel species, *Corynebacterium appendicis* sp. nov., represented by strain IMMIB R-3491^T (= DSM 44531^T = NRRL B-24151^T).

Keywords: taxonomy, phylogeny, *Corynebacterium appendicis*, 16S rRNA

During the past decade, a large number of novel *Corynebacterium* species associated with human diseases have been described (Funke *et al.*, 1997). The recognition of such species is due to the implementation of improved taxonomic methods. These include the application of chemotaxonomic methods (Schleifer & Kandler, 1972; Keddie & Cure, 1977; Collins & Cummins, 1986; Collins *et al.*, 1977, 1982a, b) and the use of miniaturized kits for biochemical characterization (Soto *et al.*, 1994) as well as the use of molecular-based approaches (Pascual *et al.*, 1995; Ruimy *et al.*, 1995), in particular 16S rRNA sequencing. Chemotaxonomically, the genus *Corynebacterium* includes species that possess wall chemotype IV (arabinose, galactose and meso-diaminopimelic acid), short-chain mycolic acids and a DNA G+C content ranging from 51 to 63 mol% (Collins & Cummins, 1986). However, there are some exceptions, with *Corynebacterium amycolatum* and *Corynebacterium kroppenstedtii* lacking mycolic acids, *Corynebacterium afermentans* and *Corynebacterium auris* exhibiting G+C contents of more than 65 mol% and a variable distribution of tuberculostearic acid within members of the genus. During the course of the characterization of bacterial isolates encountered in clinical sources using chemotaxonomic methods, we found a strain, IMMIB R-3491^T, to have chemo-

taxonomic characteristics that suggested its inclusion in the genus *Corynebacterium*. Further taxonomic and phylogenetic investigations indicated that it is different from previously described species of the genus *Corynebacterium*. Based on phylogenetic and phenotypic evidence, it is proposed that strain IMMIB R-3491^T be classified as *Corynebacterium appendicis* sp. nov.

Strain IMMIB R-3491^T was isolated from an abdominal swab of a patient with appendicitis accompanied with abscess formation. The isolate was cultured on Columbia blood agar supplemented with 5% sheep blood, brain/heart infusion (BHI) agar and trypticase soy agar (Oxoid) to determine its morphological properties. The last two media were supplemented with 1% Tween 80. Air-dried smears from BHI agar cultures were stained by the Gram and Ziehl-Neelsen methods in order to determine the Gram reaction and acid-fastness, respectively. Fermentation tests were performed using the API CORYNE system, API 20 STREP (bioMérieux) and the MINITEK system (Becton Dickinson). Enzyme reactions and acid production from carbohydrates were read after 48 and 72 h and 7 days incubation at 37 °C. Further enzyme reactions were studied by means of the API ZYM system (bioMérieux). GC analysis of fermentation products were determined with 7.0 ml culture in peptone/yeast extract/glucose broth incubated for 7 days as described by Holdeman *et al.* (1977). The isomeric form of diaminopimelic acid was determined by the methods of Becker *et al.* (1964)

The GenBank accession number for the 16S rRNA sequence of strain IMMIB R-3491^T (= DSM 44531^T = NRRL B-24151^T) is AJ314919.

and whole-cell sugars were determined by the method of Lechevalier (1968). Lipids were extracted by acid methanolysis and mycolic acids were detected with TLC as described by Minnikin *et al.* (1980). Non-hydroxylated fatty acids were purified, identified and quantified by GC as described by Yassin (1988). Phospholipids were extracted, purified and identified as described previously (Yassin *et al.*, 1993).

DNA was isolated and purified as described previously (Yassin *et al.*, 2000). DNA G+C contents were determined by HPLC (Mesbah *et al.*, 1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out using procedures described previously (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 electrophoresis of the sequencing reaction products. The 16S rDNA sequence of strain IMMIB R-3491^T, as well as those of validly described species of the genus *Corynebacterium* retrieved from GenBank, were added to the ARB database (Ludwig & Strunk, 1996) and aligned using the tools of the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using maximum-parsimony (Kluge & Farris, 1969), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary-distance matrix was calculated using the corrections of Jukes & Cantor (1969). The tree topology was evaluated according to the results of the neighbour-joining and maximum-likelihood analyses. The phylogenetic analyses were carried out using the ARB package (Ludwig & Strunk, 1996).

Strain IMMIB R-3491^T stained Gram-positive and consisted of thin, pleomorphic, coryneform cells. It was non-motile, non-spore-forming and non-acid-fast. On Columbia blood agar, colonies were very small, dry and slightly greyish in colour. Larger, slightly creamy colonies were observed on BHI agar and trypticase soy agar supplemented with 1% Tween 80. The strain grew facultatively anaerobically and was catalase- and urease-positive. The organism produced acid from glycerol. Acid production from glucose and maltose depended on the test system, as well as on the length of the incubation: with the MINITEK system, both reactions were positive after an incubation time of 3 days; with the API CORYNE system, both reactions were positive after 7 days incubation. All of the other tests of the API CORYNE, API 20 STREP, API ZYM and MINITEK systems were negative. GC analysis of the end products of glucose fermentation revealed major amounts of lactate.

Chemotaxonomically, strain IMMIB R-3491^T contained chemical markers that support its assignment to the genus *Corynebacterium*. The cell wall contains *meso*-diaminopimelic acid as well as arabinose and galactose (i.e. wall chemotype IV *sensu* Lechevalier &

Lechevalier, 1970). One-dimensional TLC analysis of whole-cell acid methanolysates of strain IMMIB R-3491^T revealed the presence of two lipid spots on the chromatogram. The lower one corresponds to corynemycolic acid, as identified by its lower R_f (0.57), and the higher spot corresponds to non-hydroxylated fatty acids. GC analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (1.24% of total fatty acids), hexadecanoate (6.54%), hexadecanoate (17.22%), octadecanoate (25.91%), octadecanoate (1.55%) and tuberculostearate (10-methyl octadecanoate, 47.51%) as major fatty acid methyl esters. Polar lipid analysis showed that strain IMMIB R-3491^T contained diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside as characteristic phospholipids, i.e. it has phospholipid type PI *sensu* Lechevalier *et al.* (1977) with no nitrogen-containing phospholipid. The result of triplicate determinations of the G+C content of the DNA of strain IMMIB R-3491^T was 65.8 ± 0.1 mol %.

The almost complete 16S rRNA gene sequence of strain IMMIB R-3491^T was determined in this study, comprising 1470 nucleotides (95.4% of the *Escherichia coli* sequence; Brosius *et al.*, 1978). The phylogenetic tree (Fig. 1) was constructed from the maximum-parsimony analysis and its topology was evaluated and corrected according to the results obtained by applying the neighbour-joining and maximum-likelihood methods. Sequences that were at least 90% complete (with regard to the *E. coli* sequence) were used for these analyses and compared with the sequences of other Gram-positive bacteria with high G+C contents. The 16S rRNA gene sequence comparison shows clearly that strain IMMIB R-3491^T is a member of the family *Corynebacteriaceae* (Stackebrandt *et al.*, 1997) and that the sequence determined contains all of the signature nucleotides designated for this lineage. Furthermore, the 16S rRNA gene sequence of strain IMMIB R-3491^T displayed high sequence similarity (91.1–97.1%) to previously described members of the genus *Corynebacterium*. Significantly lower levels of relatedness were shown to other taxa of the *Actinomycetales* (data not shown). The highest sequence similarity was shown to *C. afermentans*, *Corynebacterium coyleae* and *Corynebacterium lipophiloflavum* (97.0–97.1%). Slightly lower levels of similarity (96.6–96.7%) were shown to *Corynebacterium imitans* and *Corynebacterium mucifaciens*, whereas similarity between 96.0 and 96.3% was shown to *C. auris*, *Corynebacterium camporealensis*, *Corynebacterium riegelii*, *Corynebacterium simulans* and *Corynebacterium striatum*. The phylogenetic tree (Fig. 1) shows the position of strain IMMIB R-3491^T within the radiation of representative phylogenetic groups of the genus *Corynebacterium*. It is evident from the tree that strain IMMIB R-3491^T represents a distinct subline within the genus *Corynebacterium*. Although there is no precise correlation between the degree of 16S rRNA sequence divergence and species delineation, it is generally recognized that divergence values of 3% or

Fig. 1. Maximum-parsimony tree showing the position of strain IMMIB R-3491^T within the radiation of species of the genus *Corynebacterium*. Multifurcations indicate tree topologies that could not be resolved significantly using the neighbour-joining and maximum-likelihood algorithms. Bifurcations indicate that stable branching resulted from the various treeing algorithms. Bar, 10.0% sequence divergence.

Table 1. Characteristics that differentiate *Corynebacterium appendicis* sp. nov. from its nearest phylogenetic relatives

Species are identified as: 1, *C. appendicis*; 2, *C. afermentans*; 3, *C. coyleae*; 4, *C. lipophiloflavum*; 5, *C. imitans*; 6, *C. mucifaciens*; 7, *C. auris*; 8, *C. camporealensis*; 9, *C. riegelii*; 10, *C. simulans*; 11, *C. striatum*. (+), Reaction is positive after 7 days incubation using the API CORYNE test; w, weakly positive; v, variable; TSBA, tuberculostearic acid; ND, not determined. All species are negative for hydrolysis of aesculin.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Nitrate reduction	—	—	—	—	—	—	—	—	—	+	+
Urease	+	—	—	+	—	—	—	—	+	—	—
Pyrazinamidase	+	+	+	+	w	+	+	+	v	v	+
Alkaline phosphatase	+	+	+	+	+	+	+	+	v	+	+
Acid production from:											
Glucose	(+)	—	+	—	+	+	—	+	—	+	+
Maltose	(+)	—	—	—	+	—	—	—	+	—	—
Sucrose	—	—	—	—	w	v	—	—	—	+	v
TSBA	+	—	—	—	—	+	—	ND	—	—	ND

more are significant (Stackebrandt & Goebel, 1994). The observed 3.0% divergence between isolate IMMIB R-3491^T and *C. afermentans*, *C. coyleae* and *C. lipophiloflavum*, and even greater divergence values from other *Corynebacterium* species, are consistent with separate species status. This was also demonstrated by differences in the biochemical properties of strain IMMIB R-3491 and these phylogenetically more closely related species (Table 1).

The chemical (and morphological) data indicate clearly that the organism belongs to the genus *Corynebacterium*. The 16S rRNA gene sequencing as well as the biochemical properties indicate that it forms a novel species when compared with the type strains of validly described species of the genus *Corynebacterium*. Thus, on the basis of the results of the reported polyphasic taxonomic study, we consider that strain IMMIB R-3491^T merits classification as a novel species

of the genus *Corynebacterium*, for which the name *Corynebacterium appendicis* sp. nov. is proposed.

Description of *Corynebacterium appendicis* sp. nov.

Corynebacterium appendicis (ap.pen'di.cis. L. fem. gen. n. *appendicis* of the appendix, pertaining to appendicitis, from which the patient from whom the clinical material was taken for isolation of the organism was suffering).

Cells are Gram-positive and non-acid-alcohol-fast. Cells are thin, non-motile, non-spore-forming and pleomorphic coryneform. On Columbia blood agar supplemented with 5% sheep blood, dry, very small (diameter < 0.5 mm), slightly greyish colonies are formed after 2 days incubation at 37 °C; these colonies are non-haemolytic. Larger, slightly creamy colonies are obtained when the organism grows on BHI agar and on trypticase soy agar supplemented with 1% Tween 80. Grows facultatively anaerobically and is catalase-positive. Contains *meso*-diaminopimelic acid as wall diamino acid in addition to galactose and arabinose in whole-cell hydrolysates (i.e. cell-wall chemotype IV). Corynemycolic acids are present and the fatty acid profile contains saturated, unsaturated and tuberculostearic acids. Type PI phospholipid pattern with no nitrogen-containing compounds. Produces acid from glycerol. Acid production from glucose and maltose is positive using the MINITEK (incubation time 3 days) and API CORYNE (incubation time 7 days) systems. Acid is not produced from arabinose, arabitol, cellobiose, glycogen, inulin, lactose, mannitol, melibiose, melezitose, pullulan, salicin, sorbitol, sucrose, tagatose, trehalose, raffinose, rhamnose, ribose or xylose. Does not hydrolyse aesculin, gelatin, hippurate or starch. Displays urease, alkaline phosphatase and pyrazinamidase activities but not acid phosphatase, arginine dihydrolase, esterase (C4), ester lipase (C8), lipase (C14), α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, naphthol-AS-BI-phosphohydrolase, alanine-phenylalanine-proline arylamidase, glycyl-tryptophan arylamidase, pyroglutamic acid arylamidase, pyrrolidonyl arylamidase, valine arylamidase, cystine arylamidase, leucine arylamidase, trypsin, chymotrypsin or nitrate reductase. Acetoin production is positive but indole production is negative. Produces lactate as the major product of glucose fermentation. The G + C content is 65.8 mol % for the type strain.

The type strain, strain IMMIB R-3491^T (= DSM 44531^T = NRRL B-24151^T), was isolated from an abdominal swab of a patient with appendicitis accompanied with abscess formation.

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