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Corynebacterium appendicis sp. nov.

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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 subsline within the genus *Corynebacterium*, displaying >30% 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\textsuperscript{T} (\(=\) DSM 44531\textsuperscript{T} = NRRL B-24151\textsuperscript{T}).

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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 amylolatum* and *Corynebacterium kroppenstedti* 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\textsuperscript{T}, to have chemotaxonomic 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\textsuperscript{T} be classified as *Corynebacterium appendicis* sp. nov.

Strain IMMIB R-3491\textsuperscript{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)
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-3491T, 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-3491T 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 BHIG agar and tryptase soy agar supplemented with 1% Tween 80. The strain grew facultatively anaerobically and was Gram-positive. Acid production from glycerol, tryptophan and acid from glucose and galactose (i.e. wall chemotype IV sensu Lechevalier & Lechevalier, 1970). One-dimensional TLC analysis of whole-cell acid methanolyesates of strain IMMIB R-3491T revealed the presence of two lipid spots on the chromatogram. The lower one corresponds to coryne-myoic acid, as identified by its lower Rf (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%), hexadecenoate (17-22%), octadecenoate (25-91%), octadecanoate (1.55%) and tuberculostearate (10-methyl octadecanoate, 47-51%) as major fatty acid methyl esters. Polar lipid analysis revealed that strain IMMIB R-3491T contained diphasphatidylglycerol, phosphatidylglycerol 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-3491T was 65.8 ± 0.1 mol%.

The almost complete 16S rRNA gene sequence of strain IMMIB R-3491T 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-3491T 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-3491T 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 lipophilum (97-0–97-1%). Slightly lower levels of similarity (96-6–96-7%) were shown to Corynebacterium imitans and Corynebacterium mucificiscus, whereas similarity between 96-0 and 96-3% was shown to C. auris, Corynebacterium camporeae, Corynebacterium riegeli, Corynebacterium simulans and Corynebacterium striatum. The phylogenetic tree (Fig. 1) shows the position of strain IMMIB R-3491T within the radiation of representative phylogenetic groups of the genus Corynebacterium. It is evident from the tree that strain IMMIB R-3491T 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
Table 1. Characteristics that differentiate Corynebacterium appendicis sp. nov. from its nearest phylogenetic relatives

Species are identified as: 1, C. appendix; 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.

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The observed 3.0% divergence between isolate IMMIB R-3491T 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).
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 diaminoc acid in addition to galactose and arabinose in whole-cell hydrolysates (i.e. cell-wall chemotype IV). Corynmycolic 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, glycyll-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 = DSM 44531 = NRRL B-24151, was isolated from an abdominal swab of a patient with appendicitis accompanied with abscess formation.

References


