NOTE

***Corynebacterium aurimucosum* sp. nov. and emended description of *Corynebacterium minutissimum* Collins and Jones (1983)**A. F. Yassin,¹ U. Steiner² and W. Ludwig³¹ Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, Sigmund-Freud-Straße 25, 53127 Bonn, Germany² DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany³ Lehrstuhl für Mikrobiologie Technische Universität München, am Hochanger 4, 85350 Freising, Germany

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Two coryneform bacteria isolated from human clinical specimens were 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 sensu stricto*. Comparative 16S rRNA gene sequence analysis showed that the two strains are genealogically highly related (99.8% sequence similarity) and constitute a new subline within the genus *Corynebacterium*, with *Corynebacterium minutissimum* as their nearest phylogenetic neighbours (98.8% sequence similarity). However, DNA–DNA hybridization experiments demonstrated unambiguously that the isolates are genealogically distinct from *Corynebacterium minutissimum* (42% homology). Biochemical testing indicated that the two isolates were hardly differentiated from *Corynebacterium minutissimum*. Based on both phenotypic and phylogenetic evidence it is proposed that these isolates be classified as a new species, *Corynebacterium aurimucosum* sp. nov. The type strain of *Corynebacterium aurimucosum* is represented by strain IMMIB D-1488^T (= DSM 44532^T = NRRL B-24143^T).

Keywords: taxonomy, phylogeny, *Corynebacterium minutissimum*, *Corynebacterium aurimucosum*, 16S rRNA, DNA–DNA hybridization

The genus *Corynebacterium* was created by Lehman & Neumann (1896) to accommodate the diphtheria bacillus and some other animal-pathogenic species (Barksdale, 1970). Despite the importance of this genus in clinical and veterinary medicine, early methods used for routine identification of *Corynebacterium* species were generally unreliable. However, 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) in combination with phylogenetic approach based on 16S rRNA gene sequence analyses (Pascual *et al.*, 1995; Ruimy *et al.*, 1995) have resulted in a much improved taxonomy of the genus *Corynebacterium*. The genus belongs to the mycolic acid-containing actinomycetes – that is, to the suborder *Corynebacteriinae*

(Stackebrandt *et al.*, 1997), which forms a distinct monophyletic line that encompasses, in addition to the genus *Corynebacterium*, the genera *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania*, *Tsukamurella* and *Williamsia*. During the course of characterization of bacterial isolates encountered in clinical sources by using chemotaxonomic methods, we found two bacterial strains, namely IMMIB D-1488^T and IMMIB R-222 to have chemotaxonomic characteristics which suggested their inclusion in the genus *Corynebacterium*. Further taxonomic and phylogenetic investigations indicated that they belong to a single new species which is different from previously described species of the genus *Corynebacterium*. The name *Corynebacterium aurimucosum* is proposed for these isolates.

Strain IMMIB D-1488^T was received from a clinical laboratory for identification and strain IMMIB R-222 was isolated from blood cultures of a patient with bronchitis. The isolates were cultured on Brain-Heart Infusion (BHI) agar supplemented with 1% Tween 80,

The GenBank accession numbers for the 16S rRNA sequences of strains IMMIB D-1488^T (= DSM 44532^T = NRRL B-24143^T) and IMMIB R-222 are AJ309207 and AJ315961, respectively.

Columbia blood agar supplemented with 5% sheep blood and trypticase soy agar (Oxoid) to determine their morphological properties. 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 72 h incubation at 37 °C. Further enzyme reactions were studied by means of the API ZYM system (bioMérieux). Gas chromatographic analysis of fermentation products was determined with 7 ml of culture in peptone-yeast extract-glucose (PYG) 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 using 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 gas chromatography as described by Yassin (1988). Phospholipids were extracted, purified, and identified as described previously (Yassin *et al.*, 1993b).

DNA was isolated and purified as described previously (Yassin *et al.*, 2000). The G + C contents were determined by high-performance liquid chromatography (Mesbah *et al.*, 1989). DNA-DNA hybridization studies were carried out by using the thermal renaturation method (Yassin *et al.*, 1993a). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and the 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 the electrophoresis of the sequence reaction products. The 16S rDNA sequences of strain IMMIB D-1488^T and strain IMMIB R-222 as well as those of the validly described species of the genus *Corynebacterium* retrieved from the GenBank were added to the ARB-database (Ludwig & Strunk, 1996) and aligned using the respective tool 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). 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).

Strains IMMIB D-1488^T and IMMIB R-222 stained Gram-positive and consisted of thin pleomorphic

coryneform cells. Cells were non-motile, non-spore-forming and non acid-fast. On Columbia blood agar colonies were sticky and slightly yellow in colour. On BHI agar supplemented with 1% Tween 80 a coraloid precipitin was observed in the agar when strain IMMIB D-1488^T grows aerobically or in the presence of CO₂. This coraloid precipitin is not observed when strain IMMIB R-222 grows aerobically or in the presence of CO₂. The organisms were facultatively anaerobic and were catalase-positive. They produced acid from fructose, glucose, maltose and sucrose but not from glycogen, lactose, mannitol, mannose, ribose or xylose. Strains IMMIB D-1488^T and IMMIB R-222 hydrolysed hippurate but not aesculin, gelatin or starch. They displayed pyrazinamidase, alkaline phosphatase and leucine arylamidase activities but were negative for the other enzymic reactions of the API CORYN, API 20 STREP, API ZYM and MINITEK systems. Gas chromatographic analysis of the end products of glucose fermentation revealed major amounts of lactate.

Chemotaxonomically, strains IMMIB D-1488^T and IMMIB R-222 contained chemical markers which supports their 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 thin-layer chromatographic analysis of whole-cell acid methanolysates revealed the presence of two lipid spots on the chromatogram. The lower one corresponds to corynemycolic acid as identified by its lower *R_f* value (0.57) and the higher spot corresponds to the non-hydroxylated fatty acids. Gas chromatographic analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (1.15% of total fatty acids), hexadecanoate (0.94%), hexadecanoate (55.5%), octadecenoate (32.6%), octadecanoate (3.19%) and tuberculostearic acid (10-methyl octadecanoate 6.52%) as major fatty acid methyl esters (% fatty acids correspond to that of the type strain IMMIB D-1488^T). Polar lipid analyses showed that both strains contained diphosphatidylglycerol (DPG), phosphatidylinositol (PI) and phosphatidyl-inositol mannoside (PIM), i.e. they have phospholipid type PI *sensu* Lechevalier *et al.* (1977) with no nitrogen-containing phospholipid, as characteristic phospholipids. The result of triplicate determinations of the G + C contents of the DNA of strains IMMIB D-1488^T was 63.7 ± 0.3 mol %.

To ascertain the phylogenetic position of strains IMMIB D-1488^T and IMMIB R-222, their almost complete 16S rRNA gene sequences [1475 and 1468 nucleotides; 95.7% and 95.2%, respectively, of the *Escherichia coli* sequence (Brosius *et al.*, 1978)] were determined in this study and subjected to a comparative analyses. The 16S rRNA sequence comparison clearly shows that strain IMMIB D-1488^T and strain IMMIB R-222 are members of the family *Corynebacteriaceae* (Stackebrandt *et al.*, 1997) and that the determined sequences contain all of the

Fig. 1. Maximum-parsimony tree showing the position of strain IMMIB D-1488^T (= DSM 44532^T = NRRL B-24143^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 stable branching resulting from the various treeing algorithms. The scale bar indicates 10% sequence divergence.

signature nucleotides designated for this lineage. The high values for the 16S rRNA sequence similarity to other previously described members of the genus *Corynebacterium* (91.2 to 98.8%) support the addition of both strains to this genus. Significantly lower levels of relatedness were shown with other *Actinomycetales* taxa (data not shown). Highest sequence similarity was shown with *Corynebacterium minutissimum* (98.9% sequence similarity). The high value of 16S rRNA sequence similarity (99.8% sequence similarity which corresponds to two nucleotides differences) between strain IMMIB D-1488^T and strain IMMIB R-222 demonstrates their high genealogical relatedness. The unrooted phylogenetic tree (Fig. 1) was constructed from maximum-parsimony analysis. A total of at least 90% complete sequences (with regard to *E. coli* standard sequence) were used for these analyses. The results of maximum-parsimony analysis (Fig. 1) confirmed the association of strain IMMIB D-1488^T and IMMIB R-222 with the genus *Corynebacterium*. It was evident from the tree that strains IMMIB D-1488^T and IMMIB R-222 represents a distinct subline within the genus *Corynebacterium* that is associated with *Corynebacterium minutissimum*. These results suggest that strain IMMIB D-1488^T and IMMIB R-222 belong to a genetically distinct *Corynebacterium* species showing close relatedness with *Corynebacterium minutissimum* (98.8% sequence similarity). It has been demonstrated previously, that high levels of 16S rRNA gene sequence similarity between strains belonging to the same genus do not indicate membership of the same species (Yassin *et al.*, 1995, 1996, 2000). Here again it is clear that high

16S rRNA sequence similarities can be found between strains that have low DNA–DNA reassociation values since strains IMMIB D-1488^T and IMMIB R-222 share 98.8% gene sequence similarity but only 42% reassociation with the type strain of *Corynebacterium minutissimum*.

For further differentiation between strains IMMIB D-1488^T, IMMIB R-222 and *Corynebacterium minutissimum* their biochemical characteristics were determined in this study. The results of the biochemical tests show that strains IMMIB D-1488^T and IMMIB R-222 are hardly to differentiate from *Corynebacterium minutissimum*. All possess the same biochemical characteristics and can only be differentiated (at this time) on the basis of the hippurate hydrolysis test. However, the results of the DNA–DNA homology study (42%, which is clearly below the cut-off point of species as specified by Wayne *et al.*, 1987) support the independency of strain IMMIB D-1488^T and IMMIB R-222 from *Corynebacterium minutissimum*. A comparable case is that of *Corynebacterium propionium* and *Corynebacterium pseudodiphtheriticum*. The latter two species display 99.4% sequence similarity, possess 25% DNA–DNA relatedness and can only be distinguished on the basis of the urease test (Riegel *et al.*, 1993; Pascual *et al.*, 1995; Ruimy *et al.*, 1995). Thus on the basis of the results of the reported polyphasic taxonomic study, we consider that strains IMMIB D-1488^T and IMMIB R-222 merit classification as a new species of the genus *Corynebacterium*, for which the name *Corynebacterium aurimucosum* is proposed. In

addition we provide a complete description of *Corynebacterium minutissimum* Collins and Jones (1983).

Description of *Corynebacterium aurimucosum* sp. nov.

Corynebacterium aurimucosum (au.ri.mu.co'sum. L. n. *aurum* gold; L. neut. adj. *mucosum* slimy; N.L. adj. *aurimucosum* slimy and gold-coloured, pertaining to the appearance of colonies).

Cells are Gram-positive and non-acid-fast. They are thin, non-motile, non-spore-forming and pleomorphic coryneform. On Columbia blood agar supplemented with 5% sheep blood, the colonies are sticky and slightly yellow in colour. On trypticase soy agar they appear colourless and slimy. On BHI agar supplemented with 1% Tween 80 some strains are able to form a coraloid precipitin in agar when grown in the presence of CO₂. Growth is facultatively anaerobic. Catalase-positive. It contains *meso*-diaminopimelic acid as wall diamino acid in addition to galactose and arabinose in whole-cell hydrolysates (i.e. the cell-wall chemotype is chemotype IV). Short-chain corynemycolic acids are present. The fatty acid profile contains saturated, unsaturated and tuberculostearic acids. It has type PI phospholipid pattern with no nitrogen-containing compounds. Acid is produced from fructose, glucose, maltose and sucrose but not from adonitol, amygdalin, arabinose, cellobiose, glycerol, glycogen, inulin, lactose, mannitol, mannose, melezitose, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose or xylose. It hydrolysed hippurate but not aesculin, gelatin or starch. Positive for alkaline phosphatase, leucine arylamidase and pyrazinamidase activities, but negative for acid phosphatase, arginine dihydrolase, esterase (C4), ester lipase (C8), lipase (C14), α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucose-aminidase, α -mannosidase, α -fucosidase, naphthol-AS-bi-phosphohydrolase, pyrrolidonyl arylamidase, valine arylamidase, cystine arylamidase, trypsin, chemotrypsin, nitrate reductase and urease activities. Acetoin-positive, indole-negative. Lactate is the major product of glucose fermentation. The G + C content of the type species is 63.7 mol%. The type strain of *Corynebacterium aurimucosum* is strain IMMIB D-1488^T (= DSM 44532^T).

Description of *Corynebacterium minutissimum* Collins and Jones (1983) emend. Yassin et al. (2002)

The description below is based on the studies of the type strain DSM 20651^T (Collins & Jones, 1983) as well as our own observations.

Surface colonies on blood agar are circular, about 1 mm in diameter, low convex with entire margins, and not pigmented; the surface are shiny and moist. On BHI agar supplemented with 1% Tween 80 the organism form coraloid precipitin in agar when grown

either aerobically or in an atmosphere of CO₂. Cells are Gram-positive, non-acid-fast, non-spore-forming, non-motile and exhibit some pleomorphism. Catalase-positive. Facultatively anaerobic and non haemolytic. The cell-wall peptidoglycan contains *meso*-diaminopimelic acids. As well as arabinose and galactose as cell-wall sugars (i.e. cell-wall chemotype IV). Short-chain mycolic acids (28 to 36 carbon atoms) are present. The fatty acid profile contains saturated, unsaturated and tuberculostearic acid. The polar lipids are diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol dimannoside, and some glycolipids (i.e. phospholipid type PI). Major menaquinone components are MK-9(H₂) and MK-8(H₂). Acid is produced from fructose, glucose, maltose and sucrose. No acid is produced from adonitol, amygdalin, arabinose, cellobiose, glycerol, glycogen, inulin, lactose, mannitol, mannose, melezitose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, trehalose and xylose. Acetoin production is positive. Indole is not produced. Aesculin, gelatin and hippurate are not hydrolysed. Pyrazinamidase, alkaline phosphatase or leucine arylamidase activities are positive. Nitrate is not reduced. Urease is not produced. Acid phosphatase, arginine dihydrolase, esterase (C4), ester lipase (C8), lipase (C14), α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucose-aminidase, α -mannosidase, α -fucosidase, naphthol-AS-bi-phosphohydrolase, pyrrolidonyl arylamidase, valine arylamidase, cystine arylamidase, trypsin or chemotrypsin activities are negative. The G + C content is 58 mol% for the type strain and ranges from 56 to 58 mol% for the species. The type strain of *Corynebacterium minutissimum* is strain DSM 20651^T (= NCTC 10288^T = NRRL B-24143^T).

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