

Corynebacterium glaucum sp. nov.

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A bacterial strain, strain IMMIB R-5091^T, isolated from a cosmetic dye 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 showed that the isolate constitutes a distinct subline within the genus *Corynebacterium*, displaying >2.6% sequence divergence from established species. The isolate could be distinguished from other members of the genus *Corynebacterium* by biochemical tests. Based on both phenotypic and phylogenetic evidence, it is proposed that strain IMMIB R-5091^T (= DSM 44530^T = NRRL B-24142^T) be classified as the type strain of a novel species, *Corynebacterium glaucum* sp. nov.

The use of chemical markers (Schleifer & Kandler, 1972; Keddie & Cure, 1977; Collins *et al.*, 1977, 1982a, b) in combination with a phylogenetic approach based on 16S rRNA gene sequence analyses (Pascual *et al.*, 1995; Ruimy *et al.*, 1995) and improved phenotypic criteria (e.g. miniaturized tests) have not only resulted in a much improved taxonomy of the genus *Corynebacterium* but have also proved invaluable for the delineation of novel taxa. During the past decade, a considerable number of novel corynebacterial species have been described (e.g. Riegel *et al.*, 1993, 1997; Funke *et al.*, 1997b, 1998; Fernández-Garayzábal *et al.*, 1998; Wattiau *et al.*, 2000; Collins *et al.*, 2001; Brennan *et al.*, 2001). The vast majority of these newly described species have been isolated from human clinical and veterinary sources (Funke *et al.*, 1997b). In addition, corynebacteria are found in a broad variety of habitats such as soil, plants and food products. During the course of identification of taxonomically problematic *Actinobacteria* from different sources, using chemical and molecular taxonomic approaches, a strain designated IMMIB R-5091^T appeared to represent a novel corynebacterial species. Based on the reported findings, the name *Corynebacterium glaucum* sp. nov. is proposed for this isolate.

Strain IMMIB R-5091^T was isolated from a cosmetic dye. The isolate was cultured on brain heart infusion (BHI) agar,

BHI agar supplemented with 1% Tween 80, Columbia blood agar supplemented with 5% sheep blood and trypticase soy agar (Oxoid) to determine its 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. Fermentation tests were performed using the API CORYNE and API 20 STREP systems (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). GC analysis of fermentation products was carried out with 7.0 ml 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 was 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 GC as described by Yassin (1988). Menaquinones were extracted, purified and identified according to Collins *et al.* (1977). 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). 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

Published online ahead of print on 4 October 2002 as DOI 10.1099/ij.s.0.02394-0.

Abbreviation: BHI, brain heart infusion.

The GenBank accession number for the 16S rRNA sequence of strain IMMIB R-5091^T is AJ431634.

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 sequence reaction products. The 16S rRNA gene sequence of strain IMMIB R-5091^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). 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. Phylogenetic analyses were carried out using the ARB package (Ludwig & Strunk, 1996).

On Columbia blood agar, BHI agar, BHI agar supplemented with 1% Tween 80 and trypticase soy agar, colonies of strain IMMIB R-5091^T appeared wrinkly, dry and light-grey in colour. Cells were non-motile, non-spore-forming, dumbbell-shaped (when examined after 18 h growth) and at late stages of growth, they showed typical coryneform morphology. Cells stained Gram-positive and non-acid-fast. The organism grew facultatively anaerobically and was catalase-positive but urease-negative. It produced acid from glucose and sucrose, hydrolysed hippurate and displayed alkaline phosphatase, pyrazinamidase, ester lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities. The organism was negative for all of the other reactions of the API CORYNE, API 20 STREP, API ZYM and MINITEK systems. GC analysis of the end products of glucose fermentation revealed major amounts of lactate.

Chemotaxonomically, strain IMMIB R-5091^T contained chemical markers that support its assignment to the genus *Corynebacterium*. The cell wall contained 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 methanolsates of strain IMMIB R-5091^T revealed the presence of two lipid spots on the chromatogram. The lower one corresponds to corynemycolic acids, as identified by its lower R_f value (0.57), and the higher spot corresponds to the non-hydroxylated fatty acids. GC analysis of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (0.47% of total fatty acids), hexadecanoate (1.67%), hexadecanoate (36.71%), octadecanoate (55.25%) and octadecanoate (5.88%) as major fatty acid methyl esters. Tuberculostearic acid (10-methyl octadecanoate) was not present. Mass spectral analysis of the respiratory quinones showed that strain IMMIB R-5091^T possessed MK-7(H₂), MK-8(H₂) and MK-9(H₂), with MK-8(H₂) as the major component. Polar lipid

analysis showed that strain IMMIB R-5091^T contained diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside as characteristic phospholipids, i.e. 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-5091^T was 64.3 ± 0.7 mol%.

To ascertain the phylogenetic position of strain IMMIB R-5091^T, its almost complete 16S rRNA gene sequence (1466 nt, 95.1% of the *Escherichia coli* sequence; Brosius *et al.*, 1978) was determined in this study and subjected to a comparative analysis. 16S rRNA gene sequence comparison showed clearly that strain IMMIB R-5091^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. The high values for 16S rRNA gene sequence similarity to other previously described members of the genus *Corynebacterium* (92.3–97.4%) support the addition of strain IMMIB R-5091^T to this genus. Significantly lower levels of relatedness were shown to other taxa of the *Actinomycetales* (data not shown). Highest sequence relatedness was shown to *Corynebacterium afermentans*, *Corynebacterium riegelii*, *Corynebacterium sundsvallense* and *Corynebacterium thomsssenii* (97.2–97.4% similarity). The unrooted phylogenetic tree (Fig. 1) was constructed from maximum-parsimony analysis. Sequences that were at least 90% complete (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 R-5091^T with the genus *Corynebacterium*. It was evident from the tree that isolate IMMIB R-5091^T represents a distinct subline within the genus *Corynebacterium* that is associated with *C. afermentans*, *C. riegelii*, *C. sundsvallense* and *C. thomsssenii*. These results suggest that strain IMMIB R-5091^T belongs to a genetically distinct *Corynebacterium* species that is closely related to these four species (approx. 2.6% sequence divergence). This sequence divergence is rather low to allow the definition of a novel species, since values below 97% and/or genomic DNA reassociation values below 70% are considered as thresholds for the establishment of novel bacterial species (Stackebrandt & Goebel, 1994). However, the genus *Corynebacterium* contains a number of species for which numerous distinctive characters have been described that fully justify their classification in separate species, but that exhibit only limited 16S rRNA divergence. For instance, the 16S rRNA of *Corynebacterium mucifaciens* is 98.5% similar to that of *C. afermentans*, though distinction between the two species has been illustrated convincingly by different characters and did not require the determination of DNA hybridization values (Funke *et al.*, 1997a). Similarly, *Corynebacterium ulcerans*, *Corynebacterium pseudotuberculosis* and *Corynebacterium diphtheriae* share more than 98% 16S rRNA similarity (Pascual *et al.*, 1995). More critical examples are *Corynebacterium singulare* and *Corynebacterium minutissimum*, the 16S rRNA sequences of which are 99.1% similar,

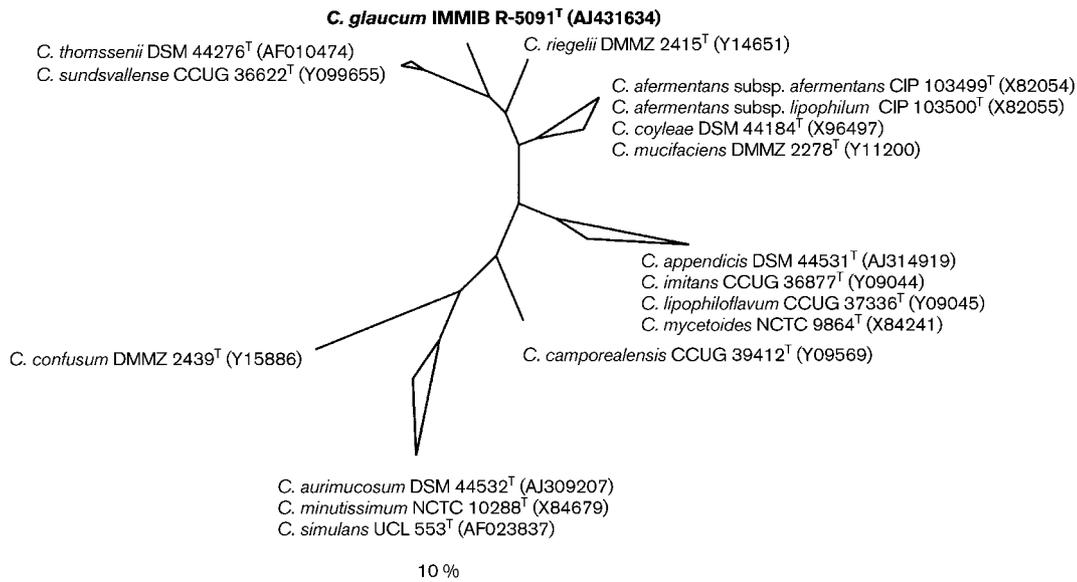


Fig. 1. Maximum-parsimony tree showing the position of strain IMMIB R-5091^T within the radiation of species of the genus *Corynebacterium*. Multifurcations indicate tree topologies that could not be resolved significantly using the neighbour-joining and maximum-likelihood algorithms. Bifurcations indicate that stable branching resulted from the various treeing algorithms. The scale bar indicates 10.0% sequence divergence.

whereas total labelled genomic DNA of the two species exhibited only 28% relatedness (Riegel *et al.*, 1997). A similar situation is found for *Corynebacterium propinquum* and *Corynebacterium pseudodiphtheriticum*, which have a 16S rRNA similarity value of 99.4% (Ruimy *et al.*, 1995) but 25% DNA–DNA relatedness (Riegel *et al.*, 1993). The 97% limit is thus not always fulfilled in the genus *Corynebacterium* and additional distinctive characters must be determined to allow the definition of a novel species. Given the 2.6–2.8% sequence divergence of strain IMMIB R-5091^T from its closest relatives *C. afermentans*, *C. riegelii*, *C. sundsvallense* and *C. thomssenii* on the one hand and the biochemical tests presented in Table 1 to discriminate

strain IMMIB R-5091^T from the latter four species on the other, we feel that it is reasonable to define a novel species. Thus, on the basis of the results of our polyphasic taxonomic study, we consider that strain IMMIB R-5091^T merits classification as a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium glaucum* sp. nov. is proposed.

Description of *Corynebacterium glaucum* sp. nov.

Corynebacterium glaucum (glau'cum. L. neut. adj. *glaucum* bluish, light-grey, pertaining to the appearance of colonies).

Cells are Gram-positive and non acid–alcohol-fast. They are non-motile, non-spore-forming and dumbbell-shaped (when examined after 18 h growth), showing typical coryneform morphology (when examined after 1 week of growth). On Columbia blood agar supplemented with 5% sheep blood, BHI agar and trypticase soy agar, colonies are light-grey in colour. Grows facultatively anaerobically and is catalase-positive. It contains *meso*-diaminopimelic acid as the wall diamino acid in addition to galactose and arabinose in whole-cell hydrolysates (i.e. cell-wall chemotype IV). Contains corynemycolic acids and the fatty acid profile contains saturated and unsaturated fatty acids. Tuberculostearic acid is absent. Contains MK-7(H₂), MK-8(H₂) and MK-9(H₂) as respiratory menaquinones, with MK-8(H₂) as the major component. Phospholipid pattern type PI, with no nitrogen-containing compounds. Produces acid from glucose and sucrose but not from arabinose, cellobiose, glycerol, glycogen, inulin, lactose, maltose,

Table 1. Differential characteristics of strain IMMIB R-5091^T and the most closely related *Corynebacterium* species

Species: 1, *C. glaucum* IMMIB R-5091^T sp. nov.; 2, *C. riegelii*; 3, *C. thomssenii*; 4, *C. sundsvallense*; 5, *C. afermentans*. All strains are negative for acid production from mannose. ND, Not determined.

Characteristic	1	2	3	4	5
Urease	–	+	+	+	–
Pyrazinamidase	–	v	+	v	–
<i>N</i> -Acetyl- β -glucosaminidase	–	–	+	ND	–
Production of acid from:					
Glucose	+	–	+	+	–
Maltose	–	+	+	+	–
Sucrose	+	–	+	+	–
Ribose	–	+	–	–	–

mannitol, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose or xylose. Hydrolyses hippurate but not aesculin, gelatin or starch. Displays alkaline phosphatase, pyrazinamidase, ester lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities but is negative for acid phosphatase, arginine dihydrolase, cystine arylamidase, esterase (C4), lipase (C14), α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, pyrrolidonyl arylamidase, valine arylamidase, trypsin, chymotrypsin, nitrate reductase and urease. Acetoin production is positive but indole production is negative. Produces lactate as the major product of glucose fermentation. The G + C content of the type strain is 64.3 mol%.

The type strain, strain IMMIB R-5091^T (=DSM 44530^T =NRRL B-24142^T), was isolated from a cosmetic dye.

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