Reclassification of *Nocardia corynebacterioides* Serrano *et al.* 1972 (Approved Lists 1980) as *Rhodococcus corynebacterioides* comb. nov.

A. F. Yassin and K. P. Schaal

Correspondence

A. F. Yassin

yassin@mibi03.meb.uni-bonn.de

Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, 53127 Bonn, Germany

The type strain of *Nocardia corynebacterioides* was the subject of a polyphasic taxonomic study. The 16S rRNA gene sequence was aligned with the sequences of representatives of the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania*, *Tsukamurella* and *Williamsia*, and phylogenetic trees were constructed by using maximum-parsimony, maximum-likelihood and neighbour-joining methods. It was evident from the phylogenetic analysis that *N. corynebacterioides* represents a distinct phyletic line within the genus *Rhodococcus*. Menaquinone analysis showed that the organism contained dihydrogenated menaquinone with eight isoprene units, MK-8(H₂), as the major isoprenologue. The genealogical evidence, together with chemotaxonomic and phenotypic data from this and previous studies, indicates that *N. corynebacterioides* DSM 20151^T (= CIP 104510^T) should be reclassified in the genus *Rhodococcus* as *Rhodococcus corynebacterioides* comb. nov.

'*Corynebacterium rubrum*' was described by Crowle (1962). Subsequently, Gordon (1966a) proposed the reclassification of this species as a member of the 'rhodochrous' group. Based on the ultrastructure, physiological characteristics, cell wall composition and phage sensitivity, Serrano *et al.* (1972) concluded that '*C. rubrum*' should be transferred to the genus *Nocardia*, and renamed the species *Nocardia corynebacterioides*. The binomial *N. corynebacterioides* was included in the Approved Lists of Bacterial Names by Skerman *et al.* (1980). In the first edition of *Bergey's Manual of Systematic Bacteriology*, Goodfellow (1986) recommended the transfer of *N. corynebacterioides* to the genus *Rhodococcus*, and considered it to be a synonym of *Rhodococcus globerulus*. This was not a nomenclaturally valid proposition, as cases of synonymy must be reported in the original literature. In a subsequent phylogenetic study of the genera *Rhodococcus* and *Nocardia*, Rainey *et al.* (1995) underpinned Goodfellow's view that the type strain of *N. corynebacterioides* belonged to the genus *Rhodococcus*, and their phylogenetic data excluded the possibility that the type strain of this species should be classified as a strain of *R. globerulus* (96.7–96.8 % sequence similarity), but that it formed a distinct phyletic line within the 16S rRNA *Rhodococcus* gene tree. Therefore, the aim of the present investigation was to

clarify the taxonomic status of *N. corynebacterioides* by using a polyphasic taxonomic approach. Based on the findings reported below, we propose that *N. corynebacterioides* should be reclassified in the genus *Rhodococcus*, as *Rhodococcus corynebacterioides* comb. nov.

The strains *N. corynebacterioides* DSM 20151^T, *Rhodococcus equi* DSM 20307^T, *Rhodococcus rhodnii* DSM 43336^T and *R. globerulus* DSM 43954^T were obtained from the German Culture Collection (DSMZ). *N. corynebacterioides* DSM 20151^T was cultured on Columbia agar (Becton Dickinson) supplemented with 5 % sheep blood and Brain Heart Infusion agar (Becton Dickinson), and was examined for pigmentation and other morphological features. Cultures were grown at 37 °C for 7 days, and observations were made at 24 h intervals. Air-dried smears at 12, 24, 48 and 72 h intervals were stained by using the Gram method, in order to determine the Gram reaction and cell morphology. The Ziehl–Neelsen method was used to determine acid-fastness. The strain was characterized physiologically by using tests to determine hydrolysis of complex substrates, as described by Gordon (1966b, 1967) and Gordon & Mihm (1957), as well as tests to determine carbon source utilization according to Yassin *et al.* (1995). The isomeric form of diamino-pimelic acid was determined by using the methods of Becker *et al.* (1964) and whole-cell sugars were determined by using the method of Lechevalier (1968). Lipids were extracted using acid methanolysis, and mycolic acids were detected by using TLC, as described by Minnikin *et al.* (1980); pyrolysis gas chromatography of the mycolate was performed according to Yassin *et al.* (1993a).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rhodococcus corynebacterioides* DSM 20151^T is X80615.

Non-hydroxylated fatty acids were purified, identified and quantified by gas chromatography, as described by Yassin (1988). Menaquinones and phospholipids were extracted, purified and identified according to Collins *et al.* (1977) and Yassin *et al.* (1993b), respectively.

Genomic DNA extraction, PCR of the 16S rRNA gene, and purification of PCR products were carried out using procedures described by 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 Analyser was used for the electrophoresis of the sequence reaction products. The 16S rRNA gene sequences of *N. corynebacterioides* DSM 20151^T, *R. equi* DSM 20307^T, *R. rhodnii* DSM 43336^T and *R. globerulus* DSM 43954^T determined in this study, as well as those of species of the genus *Rhodococcus* with validly published names retrieved from GenBank, were added to the ARB database (Ludwig *et al.*, 2004) and aligned by 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) methods. The 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 robustness of the phyletic lines were evaluated by bootstrap analyses (Felsenstein, 1985) of neighbour-joining data sets on 1000 resamplings. The phylogenetic analyses were carried out using the ARB package.

Almost complete 16S rRNA gene sequences of *N. corynebacterioides* DSM 20151^T [1494 nucleotides; 96.8% of the *Escherichia coli* sequence (Brosius *et al.*, 1978)], *R. equi* DSM 20307^T (1487 nucleotides), *R. rhodnii* DSM 43336^T (1488 nucleotides) and *R. globerulus* DSM 43954^T (1484 nucleotides) were determined in this study, and were found to be identical to the sequences of the same strains available from the public databases, under the accession numbers X80615, X80614, X80621 and X80619, respectively. Therefore, the database sequences of these species were used in the comparative analyses. The 16S rRNA gene sequence comparison clearly showed that *N. corynebacterioides* DSM 20151^T is a member of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). The determined sequence had 94.6–96.8% similarities with members of the genus *Nocardia* and 96.0–98.4% similarities with members of the genus *Rhodococcus*. The highest sequence similarity obtained was with *R. equi* and *R. rhodnii* (97.4 and 97.2% sequence similarities, respectively). The phylogenetic position of this organism, determined by using the various tree algorithms (maximum-parsimony, neighbour-joining and maximum-likelihood), revealed that *N. corynebacterioides* is closely associated with the genus *Rhodococcus* and represents a distinct subline within its members (Fig. 1). This

Fig. 1. Neighbour-joining tree showing the position of *R. corynebacterioides* DSM 20151^T within the radiation of species of the genus *Rhodococcus*. The tree was based on a comparison of sequences that were at least 90% complete (with regard to the *E. coli* sequence). Bar, 10.0% sequence divergence.

result suggests that *N. corynebacterioides* DSM 20151^T belongs to a genetically distinct *Rhodococcus* species that is closely related to *R. equi* (2.6% sequence divergence). Furthermore, the phylogenetic data exclude the possibility that the type strain of *N. corynebacterioides* is a strain of *R. globerulus* (96.8% sequence similarity), as suggested by

Goodfellow (1986), a result that is in accordance with an earlier study (Rainey *et al.*, 1995).

The type strain of *N. corynebacterioides* has morphological properties consistent with its assignment to the genus *Rhodococcus*. It is an aerobic organism that forms smooth, orange-red-pigmented colonies on Columbia agar supplemented with 5% sheep blood. The cells are rod- and coccoid-like, Gram-positive and slightly acid-alcohol-fast. Differentiating physiological properties, determined in this study, of *N. corynebacterioides* DSM 20151^T, *R. equi* DSM 20307^T, *R. globerulus* DSM 43954^T and *R. rhodnii* DSM 43336^T are given in Table 1. As can be seen from Table 1, *N. corynebacterioides* possesses unique physiological properties that clearly differentiate it from its phylogenetic relative *R. equi* and from other *Rhodococcus* species. The physiological properties of *N. corynebacterioides* are cited in detail in the description of *Rhodococcus corynebacterioides* comb. nov.

Table 1. Differential physiological characteristics of *R. corynebacterioides* DSM 20151^T and related species of the genus *Rhodococcus*

Taxa: 1, *R. corynebacterioides* DSM 20151^T; 2, *R. equi* DSM 20307^T; 3, *R. globerulus* DSM 43954^T; 4, *R. rhodnii* DSM 43336^T. All strains are positive for the utilization of acetate, adenine, glucose, testosterone and urea. All strains are negative for the utilization of adonitol, adipic acid, iso-amylalcohol, L-arabinose, lactose, maltose, raffinose, rhamnose, casein, elastin, gelatin, hypoxanthine and xanthine. +, Positive; -, negative; w, weakly utilized after 3 weeks incubation.

Characteristic	1	2	3	4
Hydrolysis of:				
Aesculin	-	-	+	-
L-Tyrosine	-	-	+	+
Utilization as sole source of carbon and energy:				
Citrate	+	-	+	-
Gluconate	+	-	+	+
Lactate	-	+	+	+
Sucrose	+	-	+	-
Trehalose	+	-	+	-
D-Xylose	w	-	-	-
myo-Inositol	+	-	-	-
Mannitol	+	-	+	+
D-Sorbitol	+	-	+	+
2,3-Butanediol	+	-	-	-
1,2-Propanediol	+	+	+	-
m-Hydroxybenzoate	-	+	-	-
p-Hydroxybenzoate	-	+	+	-
Utilization as sole source of carbon and nitrogen:				
Acetamide	-	+	-	+
L-Alanine	-	-	+	-
Proline	+	-	+	+
Serine	+	-	-	-
Ornithine	+	-	-	-

Chemotaxonomically, strain DSM 20151^T possesses chemical markers that support its assignment to the genus *Rhodococcus*. 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 of whole-cell acid methanolysates of the organism revealed the presence of two lipid spots. The lower one corresponded to mycolic acids, as identified by its R_F value (0.44), and the higher spot corresponded to non-hydroxylated fatty acids. Pyrolysis gas chromatography of the purified mycolic acid methyl esters from strain DSM 20151^T released fatty acid methyl esters of C_{14:0} and C_{16:0} as pyrolysis cleavage products. Gas chromatographic analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (4.6% of total fatty acids), pentadecanoate (3.5%), *cis*-hexadecenoate (3.3%), hexadecanoate (35.7%), 10-methyl hexadecanoate (0.8%), heptadecanoate (2.5%), heptadecanoate (2.8%), 10-methyl heptadecanoate (0.6%), octadecanoate (16.7%), octadecanoate (3.4%), tuberculostearic acid (10-methyl octadecanoate, 8.4%), eicosanoate (1.2%), docosanoate (8.5%) and tetradecanoate (5.5%) as the major cellular fatty acid methyl esters. Polar lipid analysis showed that the organism contains phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, phosphatidylglycerol and diphosphatidylglycerol as the characteristic phospholipids (i.e. phospholipid type PII *sensu* Lechevalier *et al.*, 1977). Mass spectral analysis of the respiratory quinones showed that *N. corynebacterioides* DSM 20151^T possesses dihydrogenated menaquinone with eight isoprene units [MK-8(H₂)] as the major isoprenoid quinone.

In light of the phylogenetic and chemotaxonomic evidence, the taxonomic opinion to treat *N. corynebacterioides* as a member of the genus *Rhodococcus* (Collins *et al.*, 1979; Goodfellow, 1986) has been reconsidered. As a result, it is proposed that *N. corynebacterioides* should be reclassified as *Rhodococcus corynebacterioides* comb. nov.

Description of *Rhodococcus corynebacterioides* comb. nov.

Basonym: *Nocardia corynebacterioides* Serrano *et al.* 1972 (Approved Lists 1980).

Rhodococcus corynebacterioides (co.ry.ne.bac.te.ri.oi)'des. N.L. neut. n. *Corynebacterium* bacterial genus name; Gr. suffix *-oides* similar to; N.L. adj. *corynebacterioides* similar to *Corynebacterium*).

The description is based on data from previous studies (Crowle, 1962; Gordon, 1966a; Serrano *et al.*, 1972) as well as on data obtained during this study. Smooth, orange-red-pigmented colonies are formed on agar media. Cells are rod- and coccoid-like, Gram-positive and partially acid-alcohol-fast. Grows at 22–45 °C. Contains the salient chemotaxonomic characteristic of the genus *Rhodococcus*. Mycolic acids are cleaved on pyrolysis to release fatty acids

C_{14:0} and C_{16:0} as the major cleavage products. Fatty acid profile mainly consists of straight-chain saturated, unsaturated and 10-methyl branched fatty acids. Hydrolyses adenine and testosterone, but not casein, elastin, aesculin, gelatin, guanine, hypoxanthine, tyrosine, urea or xanthine. Assimilates acetate, 2,3-butanediol, citrate, glucose, gluconate, *myo*-inositol, mannitol, 1,2-propanediol, sucrose, sorbitol and trehalose as carbon sources, but not adonitol, adipate, isoamylalcohol, arabinose, cellobiose, *meso*-erythritol, galactose, *m*-hydroxybenzoate, *p*-hydroxybenzoate, lactate, lactose, maltose, melezitose, raffinose or rhamnose. Xylose is weakly utilized as a carbon source. Utilizes proline, serine and ornithine, but not acetamide, alanine, arginine or gelatin, as simultaneous carbon and nitrogen sources.

The type strain is DSM 20151^T (= CIP 104510^T).

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