Rhodococcus triatoma sp. nov., isolated from a blood-sucking bug

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Two bacterial isolates, strains IMMIB RIV-085^T and IMMIB RIV-095, isolated from a blood-sucking bug of the genus *Triatoma*, were characterized by phenotypic and molecular taxonomic methods. Chemotaxonomic investigations revealed the presence of cell-wall chemotype IV and mycolic acids consistent with the genus *Rhodococcus*. Comparative 16S rRNA gene sequencing showed that the two isolates are genealogically highly related (100 % sequence similarity) and constitute a new subline within the genus *Rhodococcus*, with *Rhodococcus corynebacteroides* and *Rhodococcus rhodnii* as their nearest phylogenetic neighbours (98.4 and 98.3 % sequence similarity, respectively). However, DNA–DNA hybridization experiments demonstrated unambiguously that the isolates are genealogically distinct from *R. corynebacteroides* and *R. rhodnii* (32 and 43 % relatedness, respectively). The isolates could be distinguished from their phylogenetic relatives and other members of the genus *Rhodococcus* by means of biochemical tests. On the basis of both phenotypic and phylogenetic evidence, it is proposed that these isolates be classified as a novel species, *Rhodococcus triatoma* sp. nov. The type strain is strain IMMIB RIV-085^T (=CCUG 50202^T=DSM 44892^T).

The triatome vectors of Chagas' disease are obligately haematophagous insects that feed on vertebrate blood throughout their entire developmental cycle. As a result of obtaining their nutrition from a single food source, their diet is devoid of certain vitamins and nutrients. Consequently, these insects harbour populations of bacterial symbionts, within the intestinal tract, which provide the nutrients that are lacking from their diet. These bacterial symbionts are essential for the growth and development of the insect and are transmitted efficiently from adults to their progeny through coprophagy, a behaviour that is common among triatomes. Members of the genus *Rhodococcus* constitute one category of such bacterial symbionts (Dasch *et al.*, 1984).

The genus *Rhodococcus* is classified in the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997) and, together with the genus *Nocardia*, is included in the family *Nocardiaceae*. The application of molecular identification methods, particularly 16S rRNA gene sequencing, in parallel with improved phenotypic approaches has not only improved the classification of the rhodococci but also has greatly facilitated the discovery of novel species (Rainey *et al.*, 1995;

Goodfellow *et al.*, 1998; McMinn *et al.*, 2000; Gürtler *et al.*, 2004). Phylogenetic trees based on the 16S rRNA gene show that members of the genus *Rhodococcus* can be assigned to four (Rainey *et al.*, 1995), or even six (Gürtler *et al.*, 2004), subclades. The genus *Rhodococcus* has undergone considerable expansion in the past few years, and, at the time of writing, 21 species are recognized. In the present study, the taxonomic status of two isolates, IMMIB RIV-085^T and IMMIB RIV-095, which were isolated from blood-sucking bugs of the genus *Triatoma*, was determined using polyphasic taxonomic approaches. On the basis of the genotypic and phenotypic data reported here, the two isolates should be recognized as a novel species of the genus *Rhodococcus*.

Strains IMMIB RIV-085^T and IMMIB RIV-095 were cultured on Columbia agar (Becton Dickinson) supplemented with 5 % sheep blood and brain heart infusion agar (Becton Dickinson) to determine their morphological properties. The strains were biochemically characterized by using tests for the hydrolysis of complex substrates, as described previously (Gordon, 1966, 1967; Gordon & Mihm, 1957), as well as tests to determine carbon-source utilization, according to Yassin *et al.* (1995). The presence of the isomeric form of diaminopimelic acid was tested by using the methods of Becker *et al.* (1964), while whole-cell sugars were determined by the method of Lechevalier (1968). Lipids were extracted by using acid methanolysis. Mycolic acids were detected with TLC as described by Minnikin *et al.* (1980); pyrolysis GC of the mycolate was

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performed according to Yassin *et al.* (1993a). 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).

DNA was isolated and purified as described previously (Yassin *et al.*, 2000). DNA–DNA hybridization studies were carried out by using the thermal renaturation method (Yassin *et al.*, 1993b). Genomic DNA extraction, PCR-mediated amplification of 16S rRNA genes 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 rRNA gene sequences of strains IMMIB RIV-085^T and IMMIB RIV-095 as well as those (retrieved from GenBank) of *Rhodococcus* species with validly published names were added to the ARB database (Ludwig *et al.*, 2004) and aligned using the

integrated aligner software in 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. The robustness of the phyletic lines was evaluated by using bootstrap analyses (Felsenstein, 1985) of neighbour-joining datasets for 1000 resamplings. The phylogenetic analyses were carried out using the ARB package.

Strains IMMIB RIV-085^T and IMMIB RIV-095 have morphological properties consistent with their assignment to the genus *Rhodococcus*. The organisms are aerobic. They produce white to slightly creamy colonies with slightly irregular edges on Columbia agar supplemented with 5% sheep blood. Initially, the cells are thin and filamentous and stain Gram-positive and slightly acid–alcohol-fast. At a later stage of growth, the filaments fragment into rod- to

Table 1. Differential physiological characteristics of strains IMMIB RIV-085^T and IMMIB RIV-095 with respect to related species of the genus *Rhodococcus*

Taxa: 1, IMMIB RIV-085^T and IMMIB RIV-095; 2, *R. corynebacteroides* DSM 20151^T; 3, *Rhodococcus equi* DSM 20307^T; 4, *Rhodococcus globberulus* DSM 43954^T; 5, *R. rhodnii* DSM 43336^T. All of the strains are positive for assimilation of acetate and glucose and for hydrolysis of adenine, testosterone and urea. All of the strains are negative for assimilation of adonitol, adipic acid, isoamyl alcohol, L-arabinose, lactose, maltose, raffinose and rhamnose and for hydrolysis of casein, elastin, gelatin, hypoxanthine and xanthine. Symbols: +, positive result; –, negative result; w, weak utilization after 3 weeks incubation.

Characteristic	1	2	3	4	5
Hydrolysis of:					
Aesculin	–	–	–	+	–
Tyrosine	–	–	–	+	+
Urea	–	+	+	+	+
Utilization, as sole sources of carbon and energy, of:					
Citrate	+	+	–	+	–
Gluconate	–	+	–	+	+
Lactate	–	–	+	+	+
Sucrose	–	+	–	+	–
Trehalose	–	+	–	+	–
Xylose	–	w	–	–	–
<i>myo</i> -Inositol	–	+	–	–	–
Mannitol	–	+	–	+	+
Sorbitol	–	+	–	+	+
2,3-Butanediol	–	+	–	–	–
1,2-Propanediol	–	+	+	+	–
<i>m</i> -Hydroxybenzoate	–	–	+	–	–
<i>p</i> -Hydroxybenzoate	–	–	+	+	–
Utilization, as sole sources of carbon and nitrogen, of:					
Acetamide	+	–	–	–	+
Proline	–	+	–	–	+
Serine	–	+	–	–	–
Ornithine	–	+	–	–	–

To ascertain the phylogenetic position of strains IMMIB RIV-085^T and IMMIB RIV-095, their almost-complete 16S rRNA gene sequences [1486 nt; 96·3 % of the *Escherichia coli* sequence (Brosius *et al.*, 1978)], were determined in this study and subjected to comparative analyses. The 16S rRNA gene sequence comparison showed clearly that strains IMMIB RIV-085^T and IMMIB RIV-095 are members of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). The high values for the 16S rRNA gene sequence similarity to other previously described members of the genus *Rhodococcus* (96·4–98·3 %) support the addition of strains IMMIB RIV-085^T and IMMIB RIV-095 to this genus. There were significantly lower levels of similarity to other taxa of the *Actinomycetales* (data not shown). The highest sequence similarity was shown to *Rhodococcus wratislaviensis*, *Rhodococcus rhodnii* and *Rhodococcus corynebacteroides* (98·0, 98·3 and 98·4 % sequence similarity, respectively). The high value of 16S rRNA sequence similarity (100 %)

Fig. 1. Neighbour-joining tree showing the position of *R. triatomae* sp. nov. IMMIB RIV-085^T within the radiation of species of the genus *Rhodococcus*. The tree was based on a comparison of 16S rRNA gene sequences that were at least 90% complete (with regard to the *E. coli* sequence). Numbers at nodes indicate the level of bootstrap support (%) based on neighbour-joining analyses of 1000 resampled datasets; solid circles indicate that the corresponding nodes (groupings) were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 10.0% sequence divergence.

between strain IMMIB RIV-085^T and strain IMMIB RIV-095 demonstrates their high level of genealogical relatedness. The unrooted phylogenetic tree shown in Fig. 1 was constructed from neighbour-joining analysis. The results confirmed the association of strains IMMIB RIV-085^T and IMMIB RIV-095 with the genus *Rhodococcus*. It was evident from the tree that isolates IMMIB RIV-085^T and IMMIB RIV-095 represent a distinct subline, associated with *R. rhodnii*, within the genus *Rhodococcus*. These results suggest that strains IMMIB RIV-085^T and IMMIB RIV-095 belong to a genetically distinct *Rhodococcus* species that is closely related to *R. corynebacteroides* and *R. rhodnii* (1.6 and 1.7 % sequence divergence, respectively). In view of the high levels of 16S rRNA sequence similarity between strains IMMIB RIV-085^T and IMMIB RIV-095 and *R. corynebacteroides* and *R. rhodnii*, chromosomal DNA–DNA hybridization studies were performed to establish whether strains IMMIB RIV-085^T and IMMIB RIV-095 represent a distinct species. Strain IMMIB RIV-085^T displayed low levels of DNA–DNA reassociation with the *R. corynebacteroides* type strain, DSM 20151^T (32.0 %), and with the *R. rhodnii* type strain, DSM 43336^T (43.0 %); these results are below the cut-off point recommended by Wayne *et al.* (1987) for the

circumscription of bacterial genomic species, and confirm the separation of strains IMMIB RIV-085^T and IMMIB RIV-095 from their nearest phylogenetic neighbours. At present, the highest 16S rRNA gene sequence similarity value found between representatives of *Rhodococcus* species with validly published names is that between *Rhodococcus opacus* and *Rhodococcus percolatus*: the type strains of these species have a 16S rRNA gene similarity value of 99.3% and a DNA–DNA relatedness value of 71% (Briglia *et al.*, 1996).

The genotypic and phenotypic data show that strains IMMIB RIV-085^T and IMMIB RIV-095 can be distinguished from their closest phylogenetic neighbours. Therefore, on the basis of the results of the reported polyphasic taxonomic study, I consider that strains IMMIB RIV-085^T and IMMIB RIV-095 should be assigned to a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus triatomae* sp. nov. is proposed.

Description of *Rhodococcus triatomae* sp. nov.

Rhodococcus triatomae (tri.a.to'mae. N.L. gen. n. *triatomae* of *Triatoma*, a zoological genus of blood-sucking bug from which the micro-organism was isolated).

Produces white to creamy colonies with slightly irregular edges on Columbia blood agar. In the early growth phase, the cells are thin and filamentous, showing elementary branching. At a later stage of growth, the filaments fragment into rod- to coccoid-shaped elements characteristic of the genus *Rhodococcus*. The filaments as well as the rod- or coccoid-shaped elements stain Gram-positive and are slightly acid–alcohol-fast. Grows at temperatures in the range 22–42 °C. Has the salient chemotaxonomic characteristics of *Rhodococcus*. Upon pyrolysis, its mycolic acids release fatty acids C_{16:0} and C_{18:0} as the major cleavage products. The 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, citrate and glucose, but not adonitol, adipate, isoamyl alcohol, L-arabinose, 2,3-butanediol, cellobiose, *meso*-erythritol, galactose, gluconate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, myo-inositol, lactate, lactose, maltose, mannitol, melezitose, 1,2-propanediol, raffinose, rhamnose, sucrose, sorbitol, trehalose or xylose, as carbon sources for energy. Utilizes acetamide, but not alanine, gelatin, proline, serine, ornithine or arginine, as simultaneous carbon and nitrogen sources.

The type strain is strain IMMIB RIV-085^T (=CCUG 50202^T=DSM 44892^T). A reference strain is IMMIB RIV-095 (=CCUG 50203=DSM 44893).

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