Classification of strain CCM 4446T as Rhodococcus degradans sp. nov.

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Strain CCM 4446T, with notable biodegradation capabilities, was investigated in this study in order to elucidate its taxonomic position. Chemotaxonomic analyses of quinones, polar lipids, mycolic acids, polyamines and the diamino acid of the cell-wall peptidoglycan corresponded with characteristics of the genus Rhodococcus. Phylogenetic analysis, based on the 16S rRNA gene sequence, assigned strain CCM 4446T to the genus Rhodococcus and placed it in the Rhodococcus erythropolis 16S rRNA gene clade. Further analysis of catA and gyrB gene sequences, automated ribotyping with EcoRI restriction endonuclease, whole-cell protein profiling, DNA–DNA hybridization and extensive biotyping enabled differentiation of strain CCM 4446T from all phylogenetically closely related species, i.e., Rhodococcus baikonurensis, Rhodococcus qingshengii, Rhodococcus erythropolis and Rhodococcus globerulus. The results obtained show that the strain investigated represents a novel species within the genus Rhodococcus, for which the name Rhodococcus degradans sp. nov., is proposed. The type strain is CCM 4446T (=LMG 28633T).

The genus Rhodococcus is an abundant bacterial group widely distributed in various types of environments including soils, sludge, anthropogenic and marine sediments, and fresh and salt waters. Certain species are opportunistic pathogens of plants and animals, including humans. Members of the genus Rhodococcus have become important agents in the fields of environmental and industrial biotechnology and bioremediation due to their ability to degrade a remarkable range of substances, such as polycyclic and aliphatic hydrocarbons, steroids, chlorinated phenols, lignin, nitroaromatic compounds and some pesticides or polychlorinated biphenyls (Jones & Goodfellow, 2012; Martínková et al., 2009). The tolerance of members of this genus to diverse and harsh conditions is related to the high complexity, and capacity for modification, of the fatty acid composition of the cell membrane and to the usage of unique transport systems (de Carvalho et al., 2014). Rhodococci are also used for the production of enzymes for biotransformation of a number of polymers and xenobiotics (Bell et al., 1998; Warhurst & Fewson, 1994) and may be utilized as sources of biosurfactants and carotenoid pigments or as biosensors (Lang & Philp, 1998; Roach et al., 2003). The present taxonomic study deals with strain CCM 4446T (=HA1T), assigned tentatively to the genus Rhodococcus, but revealing characteristics differentiating it from species of the genus Rhodococcus with validly published names. This strain was isolated in Switzerland from a soil contaminated by organic pollutants and was originally assigned as

Abbreviations: GL, glycolipid; L, lipid; PIM, phosphatidylinositol-mannoside.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, gyrB and catA gene sequences of strain CCM 4446T are JQ776649, KP663665 and KP663666, respectively.

One supplementary table and three supplementary figures are available with the online Supplementary Material.
Arthrobacter sp. HA1 T (Scholtz et al., 1987b). It was deposited in the Czech Collection of Microorganisms (CCM) public collection in 2002. The biodegradation capacities of strain CCM 4446 T (=HA1 T) have been investigated in a number of earlier studies, which documented its significant physiological capability to reduce organic and inorganic substances from the environment. The strain has been shown to degrade polychlorinated biphenyls and low-molecular polycyclic aromatic hydrocarbons (Janáková & Vojtková, 2012; Kašáková et al., 2012). It produces dehalogenases that break the carbon-halogen bond in halogenated aliphatic compounds via the hydrolytic mechanism (Damborský et al., 1997; Damborský & Koča, 1999; Martínková et al., 2009; Poelarends et al., 2000; Scholtz et al., 1988). The strain was shown to produce 1-chloro halidohydrolase (Scholtz et al., 1987a) and to utilize 1-chloro-, 1-bromo- and 1-ido-alkanes (Scholtz et al., 1987a, b; Scholtz et al., 1988). Strain CCM 4446 T was also examined for its applicability to treating wastewater from a textile production plant containing multiple toxic metals originating from textile dying and pigmentation processes, dispersing agents, inorganic salts, dust-reducing substances, thickening agents and buffers (Vojtková et al., 2012). In the present study we determined the taxonomic position of strain CCM 4446 T.

The type and reference strains included in individual experiments were obtained from the CCM (Masaryk University, Brno, www.sci.muni.cz/ccm/).

The almost complete 16S rRNA gene sequence and sequences of the partial gene coding for the subunit B of DNA gyrase (gyrB) and the partial gene for catechol 1,2-dioxygenase (catA) involved in the metabolism of monoaromatic compounds, were determined as described by Táncsics et al. (2014). DNA amplification was performed with crude boiled cell extracts and sequencing was performed in the Eurofins MWG Operon sequencing facility (Ebersberg, Germany). The sequences obtained were compared with those of other taxa of the genus Rhodococcus retrieved from the GenBank/EMBL/DDJB database. Phylogenetic analysis was performed using MEGA version 6 software (Tamura et al., 2013). The evolutionary history was inferred using the neighbour-joining, maximum-likelihood and maximum-parsimony methods and using a bootstrap test based on 1000 replications. Maximum-likelihood and maximum-parsimony clustering confirmed the tree topologies obtained by the neighbour-joining analyses of all three genes (data not shown). The 16S rRNA, catA and gyrB gene sequence similarities were calculated using BioNumerics software (Applied Maths). Phylogenetic analysis based on the 16S rRNA gene showed that strain CCM 4446 T belongs to the Rhodococcus erythropolis 16S rRNA gene clade (Jones & Goodfellow, 2012) and placed it in a subcluster containing Rhodococcus qingshengii, Rhodococcus baikonurensis, Rhodococcus erythropolis and Rhodococcus globerulus (100 %, 99.8 %, 99.4 % and 99.0 % 16S rRNA gene sequence similarities, respectively) (Fig. 1). The phylogenetic position of strain CCM 4446 T was further assessed using catA and gyrB genes, which have been shown to be suitable alternative phylogenetic markers for differentiating between species of the genus Rhodococcus (Táncsics et al., 2014). The catA (595 bp) and gyrB (1059 bp) nucleotide sequence based phylogenetic analysis showed that strain CCM 4446 T is separate from species of the genus Rhodococcus with validly published names. The catA gene sequence similarities between CCM 4446 T and the closest species phylogenetically ranged from 90.8 % with R. globerulus to 99.4 % with R. qingshengii. Similarly, the gyrB gene sequence similarity values ranged from 88.4 % towards the R. globerulus sequence to 99.1 % towards the R. qingshengii sequence (Fig. 2).

Automated ribotyping with the EcoRI restriction enzyme was performed using the RiboPrinter Microbial Characterization System (DuPont Qualicon) in accordance with the manufacturer’s instructions. Numerical analysis and dendrogram reconstruction was carried out using BioNumerics 7.5 software (Applied Maths). The ribotype patterns were exported into the BioNumerics database using a load samples import script provided by the manufacturer. The ribotype pattern obtained from strain CCM 4446 T was clearly separated (72 % similarity) from those of the remaining type strains representing phylogenetically closely related species (Fig. S1, available in the online Supplementary Material).

Comparison of whole-cell protein profiles from strain CCM 4446 T, R. erythropolis CCM 277 T, R. globerulus CCM 8449 T, R. baikonurensis CCM 8450 T and R. qingshengii CCM 8451 T was carried out using an Agilent 2100 Bioanalyzer system with a Protein 230 kit (Agilent Technologies). Whole-cell proteins were extracted from the cells grown on Tryptone Soya agar (Oxoid) at 30 °C for 48 h. In total, 50 mg of cells were harvested, washed twice in 800 μL of PBS buffer (137 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl, 2 mM KH2PO4, pH 7.4) and finally resuspended in 600 μL PBS buffer. Subsequently, the cell suspensions were transferred into tubes containing 1 g of 0.1 mm diameter Zirconia/Silica Beads (BioSpec Products) and treated using a FastPrep-24 homogenizer for 40 s at 6 m s⁻¹. The cell lysates obtained were centrifuged and protein extracts were transferred into clean tubes and analysed immediately. Further processing of the protein extracts and analysis using the Agilent 2100 Bioanalyzer system was performed according to the protocol provided by the manufacturer. Numerical analysis of the protein patterns obtained and reconstruction of the dendrogram was carried out using BioNumerics 7.5 software (Applied Maths). Whole-cell protein profiles obtained from individual type strains were visually similar with similarity values ranging from 86 to 73 %. The dendrogram (Fig. S2) showed differentiation of strain CCM 4446 T from phylogenetically related strains of species of the genus Rhodococcus. The most similar fingerprint (86 % similarity) was that of R. globerulus CCM 8449 T.
High molecular mass genomic DNA for DNA–DNA hybridization experiments and DNA G+C content analysis was obtained using the protocol described by Gevers et al. (2001). The DNA G+C content of strain CCM 4446T was determined using the HPLC method described by Mesbah & Whitman (1989) and was 63 mol %.

DNA–DNA hybridization between strain CCM 4446T and the type strains representing the nearest phylogenetically neighbouring species was performed using the microplate method described by Ezaki et al. (1989), according to the protocols described previously (Cleenwerck et al., 2002; Goris et al., 1998). The hybridization temperature calculated from the G+C content of the strains analysed was 48 °C. The DNA–DNA relatedness percentages were calculated as means based on at least three independent hybridizations. Reciprocal reactions were performed and also considered as independent experiments. The standard deviation between reciprocal reactions was approximately 7 %, as reported by Goris et al. (1998). The DNA–DNA hybridization levels obtained between strain CCM 4446T and R. globerulus DSM 43954T, R. baikonurensis GTC 1041T, R. qingshengii CCM 4851T, R. erythropolis DSM 43066T, R. maronascens DSM 43752T, R. maanshanensis DSM 44675T, R. globerus DSM 43954T, R. baikonurensis GTC 1041T, R. degradans DSM 4446T (JQ776649) R. qingshengii DSM 4451T (AF124343) R. jostii JCM 11615T (AB046357) R. marinonascens DSM 43752T (X80617) R. maanshanensis DSM 44675T (AF416566) R. globerulus DSM 43954T (X80619) R. erythropolis DSM 43066T (X79289) R. baikonurensis GTC 1041T (AB071951) R. degradans CCM 4446T (JQ776649) R. qingshengii DSM 4451T (AF124343) R. yunnanensis DSM 44837T (AY602219) R. hoagii ATCC 7005T (X82082) R. rhodnii DSM 43336T (X80621) R. coprophilus DSM 43347T (X80626) R. ruber DSM 43338T (X80625) R. rhodochrous DSM 43241T (X79288) R. pyridinivorans DSM 44555T (AF173005) R. kroppenstedtii DSM 44908T (AY726605) R. triatoma DSM 44892T (AJ854055) R. corynebacterioides DSM 20151T (AF430066) R. degradans sp. nov. http://ijs.microbiologyresearch.org 4383

Fig. 1. Unrooted neighbour-joining tree based on 16S rRNA gene sequence comparisons showing the phylogenetic position of CCM 4446T within the genus Rhodococcus. Bootstrap probability values (percentages of 1000 tree replications) are indicated at branch points. The evolutionary distances were computed using the maximum composite likelihood method. There were a total of 1338 positions in the final dataset. Bar, 0.005 substitutions per site.
unidentified glycolipid, GL1. Also, moderate amounts of the unidentified glycolipids, GL2 and GL3, and minor amounts of PIM2 and unidentified lipids (L1, L2 and L3) were detected (Fig. S3a). A similar polar lipid profile was detected in *R. qingshengii* CCM 8451T, but in this strain only PIM1 was detectable and the glycolipids, GL1 and GL2, were absent. In addition two unidentified phospholipids, three polar lipids only detectable after total polar lipid staining (L4, L5 and L6) and one unidentified aminolipid were found (Fig. S3b), which were absent from strain CCM 4446T. Hence the polar lipid profile distinguishes strain CCM 4446T unambiguously from its nearest relative, *R. qingshengii* CCM 8451T.

The diamino acid was determined according the method described by Schumann (2011). The diamino acid of the cell-wall peptidoglycan of strain CCM 4446T was *meso*-dia-minopimelic acid.

Mycolic acids were determined according to the method of Frischmann et al. (2012). The chromatographic motility of the mycolic acids of strain CCM 4446T was slightly lower than that of *R. qingshengii* CCM 8451T, but slightly higher than that of *Rhodococcus hoogii* DSM 20307 (formerly the type strain of *Rhodococcus equi*; Kämpfer et al., 2014). Since *R. equi* was reported to contain mycolic acids consisting of 30–36 carbons (Klatte et al., 1994) the number of carbons of the mycolic acids of CCM 4446T is estimated to be 32–38.

Biomass for the detection of polyamines was harvested from the late exponential growth phase. Extraction and analysis was carried out according to Altenburger et al. (1996). Strain CCM 4446T contained (all µmol g⁻¹ dry weight) putrescine (0.1), spermidine (0.02), 1,3-diaminopropane (0.01), cadaverine (0.01), sym-homospermidine (0.01) and traces of spermine (<0.01). This pattern with very low polyamine content is similar to that reported for *Rhodococcus wratislaviensis* (Altenburger et al., 1996) and a very low polyamine content was also detected in *R. qingshengii* CCM 8451T (results not shown).

For cellular fatty acid analysis the strains were cultivated on BBL Trypticase Soy Agar plates (BD) at 28 °C for 24 h. The extraction procedure was performed according to Sasser (1990). The fatty acids were analysed by the Agilent 7890B gas chromatograph according to the standard protocol of the Sherlock MIDI Identification System (MIDI Sherlock version 6.2, MIDI database RTSBA 6.21). The fatty acid content of strain CCM 4446T corresponded with those of phylogenetically related species (Table S1).

The phenotypic characteristics of the strains were determined using conventional tube or plate tests according to Atlas (2010) and MacFaddin (2000). Conventional test characteristics were supplemented with those of the API Coryne and API ZYM microtest systems (bioMérieux). Subsequently, phenotypic fingerprinting using the Biolog system with the Gram-positive identification test panel GP2 MicroPlate (Biolog) was performed to obtain more extensive phenotypic profiles. The phenotypic characteristics of CCM 4446T are given in the species description.
Table 1. Characteristics differentiating Rhodococcus degradans sp. nov., from phylogenetically related species of the genus Rhodococcus

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Nitrate reduction</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Growth in 6.5% (w/v) NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Growth at 15 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Hydrolysis of tyrosine</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>w</td>
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<tr>
<td>Hydrolysis of DNA</td>
<td>W</td>
<td>–</td>
<td>W</td>
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<td>–</td>
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<tr>
<td>Pyrazinamidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>w</td>
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<td>Esterase (C4)</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<td>Acid from:</td>
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<tr>
<td>D-Xylose</td>
<td>+</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cellobiose</td>
<td>+</td>
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<td>Lactose</td>
<td>–</td>
<td>+</td>
<td>W</td>
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<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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</table>

Strain CCM 4446T can be differentiated from the type strains of phylogenetically closely related species using the tests listed in Table 1.

In summary, data from the present study showed that strain CCM 4446T can be distinguished both genotypically and phenotypically from all species of the genus Rhodococcus with validly published names, which demonstrates that it represents a novel species within the genus Rhodococcus. We, therefore, propose classifying this strain as Rhodococcus degradans sp. nov., with strain CCM 4446T (=LMG 28633T) as the type strain.

Description of Rhodococcus degradans sp. nov.

Rhodococcus degradans (de.gra’dans L. part. adj. degradans returning to the original order, referring to the ability of the type strain to degrade several complex organic compounds).

Cells are Gram-stain-variable rods occurring predominantly in pairs and in irregular clusters, non-spore-forming. Aerobic. Colonies on Tryptone Soya agar (Oxoid) are circular with whole margins, flat, matt with pale salmon-pink pigment and reach 1–3 mm in diameter when cultivated at 30 °C for 48 h. No haemolytic activity on sheep blood agar. The species grows at 15 and 30 °C, but it is inhibited at 37 °C. Growth in the presence of 6.5% (w/v) NaCl, but not in 10% (w/v) NaCl. Catalase, urease, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, -glucosidase and naphthol-AS-biphosphohydrolase-positive. Simmons citrate, DNase and cystine arylamidase are weakly positive. Aesculin and ONPG hydrolysis positive. Acid is aerobically produced from D-glucose, D-fructose, cellobiose, D-xylose, D-mannitol and myo-inositol when tested conventionally in a tube test, but these tests are negative in an API Coryne kit. Oxidase, arginine dihydrolase, pyrazinamidase, pyrrolidonyl arylamidase, lipase (C14), trypsin, chymotrypsin, -galactosidase, -galactosidase, -glucuronidase, N-acetyl-β-glucosaminidase, -mannosidase, -fucosidase, acetamide, Voges–Proskauer test (acetoin) and nitrate reduction negative. Hydrolysis of Tween 80, gelatin, starch, casein, tyrosine and lecithine are negative. Acid is not produced from lactose. Carbon source utilization ability via respiration, determined in Biolog GP2 MicroPlate test panels, is positive for dextrin, Tween 40, Tween 80, D-fructose, D-gluconic acid, D-glucose, maltotriose, D-mannose, D-psicose, D-ribose, trehalose, acetic acid, D-hydroxy-butyric acid, 3-ketovaleric acid, L-lactic acid, L-malic acid, pyruvatic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, L-alaninamide, L-asparagine, glycyrl-β-glutamic acid and glycerol. Negative utilization tests were for xy-cyclodextrin, -cyclodextrin, glycomen, inulin, mannann, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygaldalin, L-arabinose, D-arabitol, arbutin, D-fucose, D-galactose, D-galacturonic acid, gentiobiose, m-inositol, Z-lactose, lactulose, maltose, D-mannitol, melezitose, melibiose, 3-methyl-D-glucoside, β-methyl-D-glucoside, 3-methyl glucose, 3-methyl-D-glucoside, β-methyl-D-glucoside, 3-methyl glucose, 3-methyl-D-glucoside, p-alanine, rhamnose, raffinose, L-rhannose, salicin, sedoheptulans, D-sorbitol, stachyose, sucrose, D-tagatose, turanose, xylitol, D-xylose, -hydroxy-butyric acid, γ-hydroxy-butyric acid, p-hydroxy phenylacetic acid, 3-ketoglutaric acid, lactamide, D-lactic acid methyl ester, D-malic acid, succinamic acid, succinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, adenosine, 2-deoxyadenosine, inosine, thymidine, uridine, adenosine-5’-monophosphate, thymidine-5’-monophosphate, uridine-5’-monophosphate, D-fructose-6-phosphate, 3-D-glucose-1-phosphate, D-glucose-6-phosphate and DL-3-glycerol-phosphate. Borderline reactions were revealed for the utilization of cellobiose and N-acetyl-L-glutamic acid.

Polyamine concentrations are very low consisting of putrescine, spermidine, 1,3-diaminopropylcarboxylate, cadaverine, sym-homospermidine (0.01) and spermine. The quinone system contains predominantly menaquinone MK-8(H2), lesser amounts of MK-7(H2), MK-9(H2), MK-8 and MK-7. The major polar lipids are dihydroxybutyric acid, dihydroxybutyric acid, D-lactic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, L-alaninamide, L-asparagine, glycyrl-β-glutamic acid, glycerol. Negative utilization tests were for 3-cyclodextrin, -cyclodextrin, glycomen, inulin, mannann, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygaldalin, L-arabinose, D-arabitol, arbutin, D-fucose, D-galactose, D-galacturonic acid, gentiobiose, m-inositol, Z-lactose, lactulose, maltose, D-mannitol, melezitose, melibiose, 3-methyl-D-glucoside, β-methyl-D-glucoside, 3-methyl glucose, 3-methyl-D-glucoside, β-methyl-D-glucoside, 3-methyl glucose, 3-methyl-D-glucoside, p-alanine, rhamnose, raffinose, L-rhannose, salicin, sedoheptulans, D-sorbitol, stachyose, sucrose, D-tagatose, turanose, xylitol, D-xylose, -hydroxy-butyric acid, γ-hydroxy-butyric acid, p-hydroxy phenylacetic acid, 3-ketoglutaric acid, lactamide, D-lactic acid methyl ester, D-malic acid, succinamic acid, succinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, adenosine, 2-deoxyadenosine, inosine, thymidine, uridine, adenosine-5’-monophosphate, thymidine-5’-monophosphate, uridine-5’-monophosphate, D-fructose-6-phosphate, 3-D-glucose-1-phosphate, D-glucose-6-phosphate and DL-3-glycerol-phosphate. Borderline reactions were revealed for the utilization of cellobiose and N-acetyl-L-glutamic acid.
The type strain CCM 4446\(^\mathrm{T}\) (=LMG 28633\(^\mathrm{T}\)) was isolated from soil in Switzerland. The DNA G+C content of the type strain is 63 mol %.

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**References**


