Rhodococcus cerastii sp. nov. and Rhodococcus trifolii sp. nov., two novel species isolated from leaf surfaces

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Two Gram-positive, non-endospore-forming rods, strains C5T and T8T, were isolated from the phyllospheres of Cerastium holosteoides and Trifolium repens, respectively, and were studied in detail for their taxonomic position. 16S rRNA gene sequence analysis allocated both isolates clearly to the genus Rhodococcus. Isolate C5T was most closely related to Rhodococcus fascians and Rhodococcus yunnanensis, showing 99.2% gene sequence similarity to both species. Strain T8T revealed the highest 16S rRNA gene sequence similarity to Rhodococcus corynebacterioides (98.8%) and Rhodococcus kroppenstedti (98.6%). The quinone system of both strains was composed of dihydrogenated menaquinones with eight (major amount) as well as nine, seven and six isoprenoid units (MK-8H2, MK-9H2 MK-7H2 MK-6H2). The polar lipid profiles of strains C5T and T8T consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and one unknown phospholipid. Additionally, strain C5T contained one unknown glycolipid, and strain T8T three unknown aminolipids. The fatty acid profiles contained major amounts of C16:0, C18:1ω9c and 10-methyl C18:0, which supported the grouping of the two isolates in the genus Rhodococcus. Physiological/biochemical characterization and DNA–DNA hybridizations with the type strains of the most closely related species allowed a clear phenotypic and genotypic differentiation of both strains. For this reason, we propose strain C5T (=LMG 26203T = CCM 7906T) as the type strain of a novel species with the name Rhodococcus cerastii sp. nov., and strain T8T (=LMG 26204T = CCM 7905T) as the type strain of a second novel species with the name Rhodococcus trifolii sp. nov.

The genus Rhodococcus is classified together with the genus Nocardia in the family Nocardiaceae (Zhi et al., 2009). Since the application of 16S rRNA gene sequencing, together with improved phenotypic approaches, the number of descriptions of novel species of the genus Rhodococcus has increased dramatically. During the last decade, 18 novel species have been described (http://www.bacterio.cict.fr/qr/rhodococcus.html). The genus now harbours more than 30 species with validated names.

In this study, we describe the phenotypic and genotypic properties of two strains isolated from the leaf surfaces of different plants.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains C5T and T8T are FR714842 and FR714843, respectively.

A supplementary figure is available with the online version of this paper.
(ACGGYTACCCTGGTTCACGACTT) (Lane, 1991). The 16S rRNA genes were sequenced with standard sequencing primers. The 16S rRNA gene sequences of strains C5\textsuperscript{T} and T8\textsuperscript{T} were continuous stretches of 1337 bp and 1364 bp, respectively. Phylogenetic trees and distances were calculated using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 (Tamura et al., 2007), after alignment of sequences with CLUSTAL W (Thompson et al., 1994) and with software package ARB (version December 2004; Ludwig et al., 2004) with the corresponding SILVA SSURef 100 database (release August 2009; Pruesse et al., 2007). Distances were calculated using the Kimura two-parameter model (Kimura, 1980). Trees were reconstructed using the neighbour-joining method (Saitou & Nei, 1987) with the use of ‘default settings’ and bootstrap values were calculated based on 1000 replicates, including all available Rhodococcus species with validly published names, as well as the type strains of the type species of most genera in the suborder Corynebacterineae with validly published names at the time the calculations were performed. Additionally, trees were calculated with the maximum-parsimony method as well as with the maximum-likelihood method with fastDNAML (Olsen et al., 1994) without filters and with 30 % conservatory filter (only alignment columns in which the frequency of the most abundant nucleotide is equal to or above 30 % are included in the calculation). Tree topologies showed similar groupings for strains C5\textsuperscript{T} and T8\textsuperscript{T} and their nearest relatives. A maximum-likelihood tree without filters is shown in Fig. 1.

Sequence similarity calculations indicated that the closest relatives of strain C5\textsuperscript{T} were Rhodococcus fascians (DSM 20669\textsuperscript{T}) and Rhodococcus yunnanensis (YIM 70056\textsuperscript{T}=DSM 44837\textsuperscript{T}), both with 99.2 % sequence similarity. The closest relatives of strain T8\textsuperscript{T} were Rhodococcus corynebacterioides (NRRL B-24037\textsuperscript{T}=DSM 20151\textsuperscript{T}) with 98.8 % similarity and Rhodococcus kroppenstedtii (DSM 44908\textsuperscript{T}) with 98.6 % similarity.

DNA–DNA hybridization experiments were performed with strain C5\textsuperscript{T} and R. fascians DSM 20669\textsuperscript{T}, R. yunnanensis DSM 44837\textsuperscript{T} and Rhodococcus kytonensis CIP 109729\textsuperscript{T} and with strain T8\textsuperscript{T} and R. corynebacterioides NRRL B-24037\textsuperscript{T} and R. kroppenstedtii DSM 44908\textsuperscript{T} according to the method described by Ziemke et al. (1998). These studies resulted in DNA–DNA similarity values of 25.3 % (reciprocal 33.1 %), 47.5 % (reciprocal 56.4 %) and 33.4 % (reciprocal 41 %) in the pairing of strain C5\textsuperscript{T} with R. fascians DSM 20669\textsuperscript{T}, R. yunnanensis DSM 44837\textsuperscript{T} and R. kytonensis CIP 109729\textsuperscript{T}, respectively, and in DNA–DNA similarity values of 45.6 % (reciprocal 59.4 %) and 27.5 % (reciprocal 36.4 %) in the pairing of strain T8\textsuperscript{T} with R. corynebacterioides NRRL B-24037\textsuperscript{T} and R. kroppenstedtii DSM 44908\textsuperscript{T}, respectively.

Bacterial biomass for chemotaxonomic investigations of strains C5\textsuperscript{T} and T8\textsuperscript{T} was prepared as described previously (Martin et al., 2010). Menaquinones were extracted as described by Collins et al. (1979) and analysed by HPLC (Groth et al., 1996). Both isolates contained dihydrogenated menaquinones with eight isoprene units [MK-8(H\textsubscript{2})] as the major isoprenologue and minor amounts of MK-9(H\textsubscript{2}), MK-7(H\textsubscript{2}) and MK-6(H\textsubscript{2}) [ratio MK-8(H\textsubscript{2}) : MK-9(H\textsubscript{2}) : MK-7(H\textsubscript{2}) : MK-6(H\textsubscript{2}) = 83: 3.5: 6.6 for strain C5\textsuperscript{T} and 78: 10: 4: 5 for strain T8\textsuperscript{T}]. Polar lipids were extracted by the method of Minnikin et al. (1979) and identified according to the method of Collins & Jones (1980). Both strains exhibited a lipid pattern corresponding to phospholipid pattern type 2 sensu Lechevallier et al. (1977). Strain C5\textsuperscript{T} contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycositol, two unknown phospholipids, one glycolipid and traces of phosphatidylglycerol and phosphatidylglycositol mannoside (Fig. 2a), whereas strain T8\textsuperscript{T} contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycositol, phosphatidylglycositol mannoside, two unknown phospholipids, three unknown aminolipids and traces of phosphatidylglycerol (Fig. 2b).

Determination of the isomer of diaminopimelic acid in whole-organism hydrolysates was performed as described by Groth et al. (1996). Both isolates contained meso-diaminopimelic acid as the diagnostic diamin acid.

The occurrence of mycolic acids was determined by TLC as described by Minnikin et al. (1975) and their chain lengths as described by Klatte et al. (1994). Both strains contained series of mycolic acids similar to Rhodococcus rhodochrous strains (Alashamaony et al., 1976). Strain C5\textsuperscript{T} contained high amounts of mycolic acids with chain lengths of 44–54 carbon atoms, whereas strain T8\textsuperscript{T} contained high amounts of mycolic acids with 46–56 carbon atoms. Fatty acid analysis was performed according to the method of Kämpfer & Kroppenstedt (1996). The cells of all strains under comparison were extracted after 48 h growth on TSA in the same growth phase. Colony development of the type strains of the most closely related species was very similar, hence it can be assumed that the strains were in similar growth phases (mid-to-late-exponential phase). Fatty acids were identified with Sherlock version 2.11, TSBA40 Rev. 4.1. The fatty acid profiles of both strains were very similar to that of the closely related species of the genus Rhodococcus (Table 1). Typical fatty acid profiles, consisting of mainly C\textsubscript{16:0}, C\textsubscript{18:1}ω9c and 10-methyl C\textsubscript{18:0} were found.

Results of the comparative physiological characterization for the most closely related Rhodococcus species are given in Table 2. The NaCl requirement was determined using tryptone soy broth containing 1.0–12.0 % (w/v) NaCl (at 1.0 % intervals).

Quinone systems, fatty acid and polar lipid profiles, as well as 16S rRNA gene analyses show unambiguously that strains C5\textsuperscript{T} and T8\textsuperscript{T} are affiliated to the genus Rhodococcus. On the basis of the observed phenotypic differences, the results of the DNA–DNA pairing studies and the differences in 16S rRNA gene sequences, we propose two novel Rhodococcus species.
Fig. 1. Multiple alignment of 16S rRNA gene sequences and construction of a maximum-likelihood tree without filters were performed using the software package ARB (version December 2004; Ludwig et al., 2004) with the corresponding SILVA SSURef 100 database (release August 2009; Pruesse et al., 2007). *, These nodes were also obtained by maximum-parsimony and maximum-likelihood (without filters, with 30% conservatory filter) analysis. Bar, 0.01 nt substitutions per nucleotide position.
Two novel Rhodococcus species

**Description of Rhodococcus cerastii sp. nov.**

*Rhodococcus cerastii* (ce.ras’ti.i. N.L. n. Cerastium a scientific genus name; N.L. gen. n. cerastii of Cerastium, isolated from *Cerastium holosteoides*).

Cells are Gram-positive, aerobic, non-motile short rods (0.8–1.0 μm 2.0–3.0 μm). Colonies grown on tryptone soy agar are circular, convex and yellowish. Optimal temperature for growth is 30 °C; growth occurs at 15–50 °C, but not at 5 °C and 55 °C. Optimal pH for growth is 7–8; growth occurs at pH 6.5–10.5 and in the presence of 1–6% (w/v) NaCl (optimum, 2–3%) in tryptone soy broth. Oxidase activity is negative. Contains meso-diaminopimelic acid, mycolic acids with chain lengths of 44–54 carbon atoms and a quinone pattern with major amounts of dihydrogenated menaquinones with eight isoprene units [MK-8(H2)] and minor amounts of MK-9(H2), MK-7(H2) and MK-6(H2). The polar lipid profile consists of diphasphatidylglycerol, phosphatidylethanolamine, traces of phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, two unknown phospholipids and one unknown glycolipid. Major fatty acids are C16 : 0, C18 : 1 (9c) and 10-methyl C18 : 0. Acetate, N-acetyl-D-glucosamine, cis-aconitate, L-arabinose, p-arbutin, citrate, D-fructose, fumarate, D-galactose, D-glucose, DL-3-hydroxybutyrate, 4-hydroxybutyrate, iso-inositol, L-leucine, L-malate, maltose, D-mannitol, α-melibiose, oxoglutarate, phenylacetate, propionate, putsrescine, pyruvate, D-ribose, D-sorbilol, sucrose and trehalose are utilized as carbon sources. The utilization of N-acetyl-D-galactosamine, adipate, D-adonitol, azelate, L-alanine, β-alanine, L-aspartate, cellobiose, glucaronate, glutarate, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, D-maltitol, D-mannose, mesaconate, L-phenylalanine, suberate, L-tropolphan and D-xylose are negative, according to the method of Kämpfer et al. (1991).

The type strain was isolated from a leaf of *Cerastium holosteoides* in the Hainich-Dün region, Germany. The type strain is C5T (=LMG 26203T=CCM 7906T).

**Table 1.** Cellular fatty acid composition of strains C5T and T8T and the type strains of the most closely related species of the genus *Rhodococcus*. Strains were cultivated on TSA at 28 °C for 48 h prior to isolation of the fatty acids (identified with the Sherlock version 2.11, TSBA40 Rev. 4.1)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>C12 : 0</td>
<td>–</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<td>C13 : 0</td>
<td>0.5</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>C14 : 0</td>
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<td>3.5</td>
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<td>2.8</td>
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<td>0.5</td>
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<td>–</td>
<td>0.7</td>
<td>0.6</td>
<td>–</td>
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<td>C15 : 0</td>
<td>11.0</td>
<td>4.2</td>
<td>18.9</td>
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<td>–</td>
<td>10.5</td>
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<tr>
<td>Summed feature 3</td>
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<td>9.2</td>
<td>11.4</td>
<td>10.3</td>
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<td>36.8</td>
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<td>10-methyl C16 : 0</td>
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<td>–</td>
<td>1.2</td>
<td>0.9</td>
<td>–</td>
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<tr>
<td>C17 : 108c</td>
<td>4.6</td>
<td>1.5</td>
<td>9.1</td>
<td>1.8</td>
<td>9.3</td>
<td>12.7</td>
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<tr>
<td>C17 : 105c</td>
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<td>–</td>
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<tr>
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<td>1.8</td>
<td>–</td>
<td>1.3</td>
<td>3.2</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>10-methyl C18 : 0</td>
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<td>4.0</td>
<td>1.8</td>
<td>0.8</td>
<td>1.3</td>
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<tr>
<td>C18 : 109c</td>
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<td>14.7</td>
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<td>23.2</td>
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<td>1.3</td>
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<tr>
<td>10-methyl C18 : 0</td>
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<td>8.6</td>
<td>21.8</td>
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<td>4.3</td>
</tr>
<tr>
<td>10-methyl C19 : 0</td>
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<td>1.1</td>
<td>–</td>
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<td>–</td>
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<td>C19 : 0</td>
<td>–</td>
<td>0.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C20 : 0</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

**Fig. 2.** Two-dimensional TLC of polar lipid extracts from strains C5T (a) and T8T (b), stained with molybdatophosphoric acid. DPG, diphasphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL1, PL2, unknown phospholipids; AL, aminolipid, GL, unknown glycolipid.
Table 2. Physiological properties of strains C5\(^T\) and T8\(^T\) and the type strains of the most closely related species of the genus *Rhodococcus*

Strains: 1, C5\(^T\); 2, *R. yunnanensis* DSM 44837\(^T\); 3, *R. fascians* DSM 20669\(^T\); 4, T8\(^T\); 5, *R. cornebacterioides* NRRL B-24037\(^T\); 6, *R. kroppenstedtii* DSM 44908\(^T\) (all data from this study). All strains were positive for hydrolysis of: L-alanine-pNA, pNP-phenyl-phosphonate and bis-pNP-phosphate. All strains were negative for hydrolysis of: pNP-\(\beta\)-D-glucuronide, l-glutamate-\(\gamma\)-3-carboxy-pNA and pNP-phenyl-phosphoryl-choline. All strains were positive for the utilization of: acetate, citrate, D-fructose, fumarate, D-glucose, DL-3-hydroxybutyrate, 4-hydroxybutyrate, l-malate, malate, D-mannitol, propionate and sucrose as sole carbon sources. All strains were negative for the utilization of: N-acetyl-D-galactosamine, \(\beta\)-alanine, glucosamine, \(\gamma\)-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, 4-hydroxybutyrate, l-malate, malate, D-mannitol, propionate and sucrose as sole carbon sources. 1–6\% (w/v) NaCl (optimum, 2–3\%) in tryptone soy broth. The test for oxidase activity is negative. The major menaquinone is MK-8(H\(_2\)); minor amounts of MK-9(H\(_2\)), MK-7(H\(_2\)) and MK-6(H\(_2\)) are detected as well. The polar lipid profile consists of diphostidyglycerol, phosphatidylethanolamine, traces of phosphatidylglycerol, phosphatidyl-rhodonic acid, phosphatidylglycerol and phosphatidylglycerol mannoside, two unknown phospholipids and three unknown aminolipids. Contains meso-diaminopimelic acid and mycolic acids with chain lengths of 46–56 carbon atoms. Major fatty acids are C\(_{16:0}\), C\(_{18:1}\) 9c and 10-methyl C\(_{18:0}\). N-acetyl-D-glucosamine, L-arabinose, p-arbutin, D-glucose, D-fructose, sucrose, malate, L-rhamnose, salicin, iso-inositol, D-mannitol, putrescine, acetate, propionate, cis-aconitate, DL-3-hydroxybutyrate, 4-aminobutyrate, citrate, fumarate, 4-hydroxybutyrate, L-malate, L-proline and phenylacetate are utilized as sole sources of carbon. Negative for the utilization of: N-acetyl-D-galactosamine, celllobiose, D-mannose, \(\alpha\)-melibiose, trehalose, D-xylene, D-adenitol, D-maltitol, D-sorbitol, glucosamine, mesaconate, L-alanine, \(\beta\)-alanine, L-aspartate, L-histidine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate and 4-hydroxybenzoate. Further physiological tests (including differentiating characters determined under identical conditions) are indicated in Table 2.

The type strain was isolated from a leaf surface of *Trifolium repens* in the Hainich-Dün region, Germany. The type strain is T8\(^T\) (=LMG 26204\(^T\) = CCM 7905\(^T\)).

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**References**


