Rhodococcus sovatensis sp. nov., an actinomycete isolated from the hypersaline and heliothermal Lake Ursu

Andrés Táncsics,1* István Máthé,2 Tibor Benedek,1 Erika M. Tóth,3 Ewelina Atasayar,4 Cathrin Spröer,4 Károly Márialigeti,3 Tamás Felföldi2,3 and Balázs Kriszt5

Abstract

A Gram-stain-positive, strictly aerobic, mesophilic bacterium, designated H004T, was isolated from a water sample of the hypersaline and heliothermal Lake Ursu, Sovata, Romania. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain H004T formed a distinct phylogenetic lineage within the genus Rhodococcus. It shared the highest 16S rRNA gene sequence similarity with Rhodococcus yunnanensis YIM 70006T (98.80%), followed by Rhodococcus fascians DSM 3623T (98.73%), Rhodococcus cercidiphylli YIM 65003T (98.73%), Rhodococcus cerastii C5T (98.58%) and Rhodococcus kyotonensis DS472T (98.53%). The alkB-based phylogenetic analysis further confirmed that this strain constitutes a highly unique lineage within the genus. Chemotaxonomic characteristics, including the predominant fatty acids acids C15:0, C18:1ω9c, C19:1ω11c/C19:1ω9c and C16:1ω7c/iso-C15:0 2-OH, the major quinone MK-8(H2), the presence of mycolic acids and cell-wall chemotype IV were also consistent with the properties of members of the genus Rhodococcus. The DNA G+C content of strain H004T was 65.4 mol%. The results of DNA-DNA hybridization analyses with the closest relatives, in combination with the alkB-based phylogenetic analysis, as well as the chemotaxonomic and physiological data, demonstrated that isolate H004T represents a novel species of the genus Rhodococcus, for which the name Rhodococcus sovatensis sp. nov. is proposed. The type strain is H004T (≡DSM 102881T=NCIMB 0.02632T).

Members of the genus Rhodococcus are primarily isolated from pristine and hydrocarbon-contaminated soils. In addition, several species of the genus were described from freshwater and marine ecosystems [1]. The fact that endophytic, insect- and phyllosphere-associated species can be also found in the genus proves that rhodococci are ubiquitous [1–3]. Recently, the benefits of using alternative marker genes in the taxonomy of Rhodococcus has been shown by several studies [4–7]. However, the alkB gene (key gene of aerobic alkane degradation) is the only one of which applicability as a phylogenetic marker gene was analysed for the entire genus [6]. It was shown that a certain type of alkB gene is present in almost every member of the genus and is applicable to assess whether a new Rhodococcus isolate represents a novel species. The present study aims to describe a novel species of the genus Rhodococcus using polyphasic characterization supplemented with an alkB-based phylogenetic analysis.

Strain H004T was isolated from a water sample taken into sterilized screw-capped bottles from a 4 m depth of the hypersaline and heliothermal Lake Ursu (Sovata, Romania; detailed site description is available in [8, 9]), using the standard dilution plating technique on Halomonas agar medium [10]. The plates were incubated for one week at 28°C for the isolation of bacteria. Short-term maintenance of the isolate was performed on trypticase soy agar (TSA; BBL) at 28°C for 7–10 days.

The type strains Rhodococcus yunnanensis JCM 13366T and Rhodococcus kyotonensis JCM 23211T were used as reference strains for comparison of phenotypic properties of strain H004T in this study under the same laboratory conditions. Colony morphology of strain H004T was directly observed on TSA medium. Cell morphology of the isolate was examined under a light microscope at ×1000 magnification using cells grown for 8, 24, 30, 46, 54, 71 and 79 h at 28°C on TSA.

Author affiliations: 1Regional University Center of Excellence in Environmental Industry, Szent István University, Páter K. u. 1, H-2100 Gödöllő, Hungary; 2Department of Bioengineering, Sapientia Hungarian University of Transylvania, Piaţa Libertăţii 1, R-530104, Miercurea Ciuc, Romania; 3Department of Microbiology, Eötvös Loránd University, Pázmány P. sétány 1/C, H-1117 Budapest, Hungary; 4DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstrasse 7B, D-38124, Braunschweig, Germany; 5Department of Environmental Protection and Environmental Safety, Szent István University, Páter K. u. 1, H-2100 Gödöllő, Hungary.

*Correspondence: András Táncsics, tancsics.andras@fh.szie.hu
Keywords: Rhodococcus; alkB; Alkane 1-monoxygenase; Hypersaline; Heliothermal.
Abbreviation: DDH, DNA–DNA hybridization.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and alkB sequences of strain H004T are KU189221 and KU189222, respectively.
One supplementary figure is available with the online Supplementary Material.
medium. The shape, size and arrangement of the cells were studied in Hucker’s Gram-stained smears [11]. Motility was examined by phase-contrast microscopy in wet-mount preparations and using deep semi-solid TSA medium with 0.3% (w/v) agar, respectively. The following physiological and biochemical tests were performed according to the protocols of Barrow and Feltham [11]: methyl-red and Barritt’s Voges-Proskauer tests, nitrate reduction to nitrite or nitrogen, urease activity, Baird-Parker’s phosphatase activity, production of hydrogen sulphide from cysteine, production of indole from tryptophan, and hydrolysis of casein, gelatin, aesculin and Tween 80. Catalase activity was determined by bubble production with $H_2O_2$ (3%, v/v), and oxidase activity was tested using 1% (w/v) tetramethyl-p-phenylenediamine oxalate. Acid production from glucose was checked by the OF test according to the method of Hugh and Leifson [12]. Growth at different temperatures (from 5 to 45°C) was determined by using TSA medium, while pH tolerance (pH 3–11, using increments of 1 pH unit; the pH was adjusted prior to sterilization by adding 0.1 M HCl or 0.1 M NaOH) was determined using TSB medium. Tolerance of salinity was determined by inoculating the strain into TSB medium supplemented with 0–12% (w/v) NaCl at 1% intervals. During pH and NaCl tolerance tests, growth of strains was followed by measuring OD at 600 nm. API 50CH, API20NE and API ZYM strips (bioMerieux) were used to determine physiological and biochemical characteristics according to the manufacturer’s instructions.

Differential phenotypic characteristics of strain H004T together with those of the phylogenetically related type strains of *R. yunnanensis* JCM 13366T and *R. kyotonensis* JCM 23211T are presented in Table 1. Growth temperature and NaCl tolerance ranges of strain H004T are similar to those of the above-mentioned type strains. However, enzyme activity and carbon source profiles of strain H004T markedly differ from those of *R. yunnanensis* JCM 13366T and *R. kyotonensis* JCM 23211T. Cells of strain H004T are 1.2–2.2 µm long rods after incubation for 8 h, occurring as single cells or pairs. Hyphae-like structures are not observed but cells are remarkably shortened after longer incubation times (1–1.5 µm long cocccobacilli after 79 h incubation).

To determine the cellular mycolic and fatty acids composition, the cells were grown for 4 days (early stationary growth phase) at 28°C on TSA (BBL). The mycolic and fatty acids methyl esters were obtained from cells scraped from Petri dishes by saponification, methylation and extraction using minor modifications of the method of Miller [13] and Kuykendall et al. [14]. The obtained methyl esters mixtures were separated using gas chromatography (Sherlock Microbial Identification System; MIDI). Fatty acids were identified using Sherlock version 6.1 (Method TSBA40, library TSBA40 4.10). The chromatograms for mycolic acids were evaluated manually by comparing to the chromatograms of standard samples. The DNA G+C content of strain H004T was determined from bacterial cells disrupted by using a Constant Systems TS 0.75 KW French press (IUL Instruments). After purification of the DNA on hydroxyapatite according to the procedure of Cashion et al. [15], the DNA was degraded to nucleosides using P1 nuclease and bovine alkaline phosphatase as described by Membah et al. [16]. The nucleosides were separated by reversed-phase HPLC by the method described by Tamaoka and Komagata [17]. The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine.

For the analysis of diamino acids, whole-cell sugars, polar lipids and menaquinones, freeze-dried cells were used. The diamino acids were identified in the whole-cell hydrolysates (6 M HCl, 100°C, 18 h) and detected by thin-layer chromatography (TLC) on cellulose plates by using the solvent system of Rhuland et al. [18]. The sugars in the whole-cell hydrolysates (2 M H$_2$SO$_4$, 100°C, 2 h) were analysed by TLC on cellulose plates according to Staneck and Roberts [19]. The polar lipids and menaquinones were extracted based on the modified procedure of Bligh and Dyer [20]. Total lipid material was detected using molybdocephosphoric acid spray reagent and specific functional groups were detected with molybdenuim blue (for phospholipids), ninhydrin (for amino lipids), anise aldehyde (for phosphatidylinositol mannosides) and Dragendorff’s reagent (for phosphatidylcholine). The menaquinones were analysed by LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 x 125 mm, 3 µm, RP18) using methanol: heptane (9:1, v/v) as the eluant.
The cellular membrane of strain H004\(^{T}\) contained C\(_{15:0}\), C\(_{16:0}\)ω9c, C\(_{19:1}\)ω11c/C\(_{19:1}\)ω9c and C\(_{16:0}\)ω7c/iso-C\(_{15:0}\) 2-OH as major fatty acids. The significantly larger amounts of C\(_{15:0}\) and C\(_{19:1}\)ω11c/C\(_{19:1}\)ω9c as well as the smaller amounts of C\(_{16:0}\) and C\(_{18:0}\) 10-methyl (TBSA) obviously differentiated strain H004\(^{T}\) from the type strains of \(R.\) yunnanensis and \(R.\) kyotonensis (Table 2). The DNA G+C content of strain H004\(^{T}\) was 65.4 mol%. The diamino acid of the cell-wall peptidoglycan of strain H004\(^{T}\) was meso-diaminopimelic acid which is characteristic to the genus \(R.\) cerastii. Whole-cell hydrolysates were rich in arabinose and galactose (cell wall chemotype IV sensu [22]). The phosholipids contained diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol phospholipids contained diphosphatidylglycerol, phosphatidylinositol-phosphatidylinositol mannosides, an unknown aminolipid and four unknown lipids (Fig. S1, available in the online Supplementary Material). The quinone system of strain H004\(^{T}\) was composed of menaquinone MK-8\((\text{H}_2)\) as the predominant respiratory quinone (74.4 %) with minor amounts of MK-7\((\text{H}_2)\) (14.4 %) and MK-6\((\text{H}_2)\) (11.2 %). Strain H004\(^{T}\) contains mycolic acids with chain lengths of 46–56 carbon atoms.

The 16S rDNA gene of strain H004\(^{T}\) was amplified using the universal bacterial primers E27F and E1492R [23]. The almost-complete 16S rDNA gene sequence of the strain was determined by using BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Sequencing primers were E27F, E338F, E786F and E1492R [23]. Sequencing products were separated on a Model 3130 Genetic Analyzer (Applied Biosystems) and closely matched to the type strains of members of the genus \(R.\) Rhodococcus. Phylogenetic trees were reconstructed using the neighbour-joining [25] and maximum-likelihood [26] methods with Kimura’s two-parameter calculation model and the maximum-parsimony algorithm [27] using MEGA software version 6.0 [28]. Tree topologies and distances were estimated by bootstrap analysis based on 1000 replicates. For DNA–DNA hybridization (DDH) experiments, bacterial cells were disrupted by using a Constant Systems TS 0.75 KW french press (IUL Instruments) and the DNA in the crude lysate was purified by chromatography on hydroxylapatite as described by Cashion et al. [15]. DDH analyses were carried out as described by De Ley et al. [29] under consideration of the modifications described by Huss et al. [30] using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-controlled 6×6 multicell changer and a temperature controller with in situ temperature probe (Varian).

Phylogenetic analysis based on 16S rDNA gene sequences indicated that strain H004\(^{T}\) formed a distinct phylectic lineage within the genus \(R.\) Rhodococcus (Fig. 1.)

### Table 2. Cellular fatty acid contents of strain H004\(^{T}\) and closely related species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_9:0)</td>
<td>TR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C(_10:0)</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C(_11:0)</td>
<td>TR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C(_12:0)</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C(_13:0)</td>
<td>1.9</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C(_14:0)</td>
<td>2.9</td>
<td>9.0</td>
<td>5.3</td>
</tr>
<tr>
<td>C(_15:0)</td>
<td>3.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C(_16:0)</td>
<td>24.4</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>C(<em>16:1)ω7c/iso-C(</em>{15:0})2-OH*</td>
<td>10.1</td>
<td>8.8</td>
<td>9.8</td>
</tr>
<tr>
<td>C(_16:0)</td>
<td>8.6</td>
<td>34.0</td>
<td>34.5</td>
</tr>
<tr>
<td>C(_16:0)10-methyl</td>
<td>–</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C(_17:0)ω8c</td>
<td>7.6</td>
<td>1.0</td>
<td>2.1</td>
</tr>
<tr>
<td>C(_17:1)ω5c</td>
<td>–</td>
<td>2.0</td>
<td>–</td>
</tr>
<tr>
<td>C(_17:0)</td>
<td>3.0</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>C(_17:0)10-methyl</td>
<td>2.7</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>C(_18:1)ω9c</td>
<td>12.1</td>
<td>11.3</td>
<td>19.6</td>
</tr>
<tr>
<td>C(_18:0)</td>
<td>3.4</td>
<td>TR</td>
<td>–</td>
</tr>
<tr>
<td>C(_18:1)10-methyl (TBSA°)</td>
<td>6.2</td>
<td>16.6</td>
<td>14.7</td>
</tr>
<tr>
<td>C(<em>19:1)ω11c/C(</em>{19:1})3c-9t ‡</td>
<td>10.3</td>
<td>2.4</td>
<td>2.3</td>
</tr>
<tr>
<td>C(_19:0)</td>
<td>TR</td>
<td>1.70</td>
<td>TR</td>
</tr>
<tr>
<td>C(_17:0)10-methyl</td>
<td>–</td>
<td>1.0</td>
<td>TR</td>
</tr>
<tr>
<td>C(_20:0)ω6,9c</td>
<td>–</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C(_20:0)</td>
<td>1.6</td>
<td>TR</td>
<td>TR</td>
</tr>
</tbody>
</table>

* Summed feature 3.
† Tuberculostearic acid.
‡ Summed feature 6.

The overall topology of the maximum-likelihood tree was similar to that of the neighbour-joining and maximum-parsimony trees. Comparative 16S rDNA gene sequence analysis revealed that strain H004\(^{T}\) belongs to a subcluster containing \(R.\) yunnanensis YIM 70056\(^{T}\) with 16S rDNA gene similarity of 98.80 %, followed by \(R.\) fascians DSM 3623\(^{T}\), \(R.\) ceridiphylli YIM 65003\(^{T}\), \(R.\) cerastii C5\(^{T}\) and \(R.\) kyotonensis DSM 472\(^{T}\) with 16S rDNA gene similarities of 98.73, 98.73, 98.58 and 98.53 %, respectively. The phylogenetic position of strain H004\(^{T}\) was further assessed by using the alkB gene (the universal alkB gene-type of rhodococci) which was shown to be a highly suitable alternative phylogenetic marker differentiating species of the genus \(R.\) Rhodococcus [6]. The alkB-based tree topologies further confirmed the phylogenetic position of strain H004\(^{T}\), which formed a robust and highly distinct clade with \(R.\) kyotonensis and \(R.\) yunnanensis (Fig. 2). The highest alkB sequence similarity was observed with \(R.\) kyotonensis DSM 472\(^{T}\) (83.75 %), and gradually lower similarities...
Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain H004T and related taxa. Bootstrap values are shown as percentages of 1000 replicates; only values >50% are shown. Type strains of species.
were observed with *R. cercidiphylli* YIM 65003T (81.25 %), *R. yunnanensis* YIM 70056T (81.24 %), *R. fascians* LMG 3623T (80.23 %) and *R. cerastii* C5T (78.47 %). It was shown earlier that the universal *alkB* gene of rhodococci has a larger phylogenetic resolution than the 16S rRNA gene [6]. It was also observed that low *alkB* sequence similarity between closely related species of the genus *Rhodococcus* accompany a low DDH value, which is particularly true in case of the subclade containing *R. yunnanensis*, *R. kyotonensis*, *R. cerastii*, *R. cercidiphylli* and *R. fascians* [6]. In case of strain H004T, the considerably low *alkB* gene sequence similarity with its closest relatives justifies the species level differentiation. Nevertheless, DDH was performed between strain H004T and type strains representing the phylogenetically closely related species. The DDH values obtained between strain H004T and type strains of *R. yunnanensis*, *R. fascians*, *R. cercidiphylli*, *R. cerastii* and *R. kyotonensis* were 15, 16, 13, 21 and 25 %, respectively. Considering the threshold value of 70 % DNA–DNA relatedness for the delineation of bacterial species [31], it can be stated that strain H004T does not belong to any of the above-mentioned species.

In essence, the phenotypic, phylogenetic and chemotaxonomic characteristics confirm that strain H004T represents a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus sovatensis* sp. nov. is proposed.

**Fig. 2.** Unrooted maximum-likelihood tree based on *alkB* gene sequences showing the phylogenetic position of strain H004T within the genus *Rhodococcus*. Bootstrap values are shown as percentages of 1000 replicates; only values >50 % are shown. Asterisks indicate branches that occurred with every tree-making algorithm used in the study. Bar, 1 substitutions per 100 nt positions.
DESCRIPTION OF RHODOCoccus sovataNsis SP. NOv.

Rhodococcus sovataNsis (so.va.ten’sis. N.L. masc. adj. sovanNsis pertaining to Sovata, Romania, where Lake Ursu is located).

Gram-stain-positive, aerobic, non-motile, and mesophilic actinobacterium. Catalase-positive and oxidase-negative. Colonies on TSA medium are orange-pigmented, smooth and opaque. The temperature range for growth is 10–35 °C, with an optimum temperature of 28–30 °C; growth cannot occur at 37 °C. Growth occurs between pH 6–10, with an optimum pH of around 7.0. The NaCl tolerance range in TSB medium is 0–9%. Nitrate and nitrite are not reduced. Positive results for the activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, α-glucosidase and hydrogen sulphide production, but negative results for trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, urease, gelatinase, casease, amylase, hydrolysis of Tween 80 and aesculin, indole production, oxidative and fermentative acid production from D-glucose and Voges–Proskauer test. In the API 50 CH and API 20 NE tests, utilizes d-arabitol, d-fructose, D-glucose, D-mannitol, D-mannose, D-ribose, D-sorbitol, trehalose, malic acid, potassium gluconate, salicin, trisodium citrate and xylitol. The diamino acid in the peptidoglycan is meso-diaminopimelic acid, and arabinose and galactose are the dominant sugars in the whole-cell hydrolysates. The percentage of major menaquinone MK-8(H2) is 74.4%, that of minor menaquinones MK-7(H2) and MK-6 (H2) is 14.4 and 11.2%, respectively. The mycolic acids range from C46 to C56. Predominant phospholipids are diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylinositol. The major fatty acids are C15:0, 3-OH; C16:0 3-OH; C17:0 3-OH; and C18:0. The type strain, H004T (=DSM 102881T=NCAIM B.02632), was isolated from a water sample of Lake Ursu, Sovata, Romania. The DNA G+C content of the type strain is 65.4 mol%.

Funding information
This work was supported by the Romanian National Authority for Scientific Research CNCS-UEFISCDI (grant PN-II-PD-TE-2012-3-0319) and by the Hungarian grant Research Centre of Excellence – 11476/3/2016/FEKUT.

Acknowledgements
We would like to thank Bettina Sträuber (DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstrasse 7B, D-38124, Braunschweig, Germany) for excellent technical assistance.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.