Taxonomic Note

Sequence analysis of 16S rRNA, *gyr*B and *cat*A genes and DNA–DNA hybridization reveal that *Rhodococcus jialingiae* is a later synonym of *Rhodococcus qingshengii*

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The results of 16S rRNA, *gyr*B and *cat*A gene sequence comparisons and reasserted DNA–DNA hybridization unambiguously proved that *Rhodococcus jialingiae* Wang *et al.* 2010 and *Rhodococcus qingshengii* Xu *et al.* 2007 represent a single species. On the basis of priority *R. jialingiae* must be considered a later synonym of *R. qingshengii*.

A carbendazim-degrading isolate from a carbendazim waste-water plant in China has been described as a novel member of the genus *Rhodococcus* and named as *Rhodococcus jialingiae* (Wang *et al.*, 2010). Its closest relative has been reported to be *Rhodococcus qingshengii*, another carbendazim-degrading member of the genus, isolated from carbendazim-contaminated soil in China (Xu *et al.*, 2007). As they share almost identical 16S rRNA gene homology (99.8 %) the species level differentiation of these strains was based on their low DNA–DNA hybridization (DDH) value, which was reported to be 27.7 % (Wang *et al.*, 2010). Since the routine identification of environmental isolates of members of the genus *Rhodococcus* is still based on the determination of the 16S rRNA gene sequence, this made the identification of environmental isolates of members of the genus *Rhodococcus* closely related to the *R. qingshengii*–*R. jialingiae* lineage ambiguous. Moreover, the 0.2 % difference in the 16S rRNA gene sequence can be observed upstream of the 1492r primer site. In the case of *R. jialingiae* 13 nt can be found at this site on the 16S rRNA gene sequence deposited in the GenBank database (GenBank accession number: DQ185597), while in the case of *R. qingshengii*, only 3 nt can be found in this

position (GenBank accession number: DQ090961) and these nucleotides cause the 0.2 % difference.

During our recent studies, we have observed that environmental isolates of members of the genus *Rhodococcus* most closely related to the *R. qingshengii*–*R. jialingiae* lineage can be isolated frequently from hydrocarbon-contaminated soils (Máthé *et al.*, 2012; Benedek *et al.*, 2013). At first, we decided to look for a marker gene other than the 16 rRNA gene that would be suitable for easily differentiating these two species. Later, our results led us to reassess the analysis of DDH between the type strains of *R. qingshengii* and *R. jialingiae* and as a consequence to suggest the reconsideration of the present taxonomic status of *R. jialingiae*.

Rhodococcal type strains used in this study were obtained from the RIKEN Bioresource Center – Japan Collection of Microorganisms and from the DSMZ – German Collection of Microorganisms and Cell Cultures. Environmental isolates used in this study originated from the culture collections of the Department of Environmental Protection and Environmental Safety, Szent István University, Gödöllő, Hungary and the Bioengineering Department of Sapientia Hungarian University of Transylvania, Miercurea Ciuc, Romania. The following type strains were included in this study: *R. jialingiae* DSM 45257[T], *R. qingshengii* DSM 45222[T], *Rhodococcus baikonurensis* DSM 44587[T], *Rhodococcus erythropolis* JCM 3201[T] and *Rhodococcus globerulus* JCM 7472[T]. The environmental isolates related

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Abbreviation: DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyr*B and *cat*A gene sequences of members of the genus *Rhodococcus* obtained in this study are KF790905, KF360059–KF360061, KF374690–KF374699 and KF500428–KF500438.

to the *R. qingshengii*–*R. jialingiae* lineage were the following (GenBank accession numbers are given in parentheses): *Rhodococcus sp.* BBG1 (HE820128), *Rhodococcus sp.* RGN4 (HE801274), *Rhodococcus sp.* K5 (KF790905), *Rhodococcus sp.* PT2-14B (KF360060), *Rhodococcus sp.* PT3-14 (KF360061) and *Rhodococcus sp.* Ba49 (KF360059). All strains were maintained on DSMZ medium 1 (nutrient agar) at 28 °C.

Genomic DNA was extracted from pure cultures by using the UltraClean Microbial DNA Isolation kit (MoBio) according to the instructions of the manufacturer. These genomic DNA samples were used as templates for the amplification of target genes.

For the amplification of the 16S rRNA gene sequence the primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525r (5′-AAGGAGGTGWTCCARCC-3′) were used to reveal the sequence stretch upstream of the 1492r primer site. The applied annealing temperature was 52 °C.

To design PCR primers for the detection of gyrase B (*gyrB*) genes of members of the *erythropolis* clade of the genus *Rhodococcus* the following complete *gyrB* nucleotide sequences were retrieved from NCBI GenBank: *Rhodococcus jostii* RHA1 (CP000431), *Rhodococcus opacus* B4 (AP011115), *Rhodococcus equi* 103S (FN563149) and *R. erythropolis* PR4 (AP008957). The forward primer RHO-gyrBF was a 20-mer oligonucleotide (5′-GGCGGCAAGTTCGACTTCGA-3′) targeting the amino acid sequence GGKFDSD (*R. erythropolis* PR4 gyrB amino acid position 109–115). The reverse primer RHO-gyrBR was a 23-mer oligonucleotide (5′-GCCTT-CTCGACGTTGATGATC-3′) targeting the amino acid sequence KIINVEKA (*R. erythropolis* PR4 gyrB amino acid position 486–493). The expected length of the amplified fragment was approximately 1154 bp. The applied annealing temperature was 68 °C.

For the amplification of catechol 1,2-dioxygenase (*catA*) primers RHO-F (5′GCCGCCACCGACAAGTT-3′) and RHO-R (5′-CACCATGAGGTGCAGGTG-3′) were used (Táncsics *et al.*, 2008). The applied annealing temperature was 58 °C.

PCRs were performed in 50 μl reactions containing 5 μl 10 × PCR buffer, 0.3 μM of each primer, 0.2 mM of each dNTP, 1 μl extracted DNA, 1 U DreamTaq DNA Polymerase (Thermo Scientific) and nuclease-free water up to the final reaction volume. The amplification conditions were as follows: 95 °C for 3 min, then 32 cycles of 94 °C for 30 s, the appropriate annealing temperature for 30 s and 72 °C for 1 min, then a final extension at 72 °C for 10 min. All amplification products were analysed by electrophoresis on 1 % (w/v) agarose gel stained with ethidium bromide.

The PCR products were purified by using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to the instructions of the manufacturer and sequenced directly or cloned in *Escherichia coli* TOP10 cells by using the pCR 2.1 cloning vector system (Invitrogen). Amplicons were sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Life Technologies). Cycle

sequencing products were analysed with a model 3130 Genetic Analyzer (Life Technologies). The 16S rRNA, *gyrB* and *catA* gene sequences obtained in this study were deposited in the GenBank nucleotide sequence database under accession numbers KF790905, KF360059–KF360061, KF374690–KF374699 and KF500428–KF500438. Sequence reads were assembled in MEGA5 (Tamura *et al.*, 2011) then aligned by using the CLUSTAL W algorithm. Neighbour-joining trees (Saitou & Nei, 1987) were reconstructed in MEGA5, with 10 000 bootstrap replicates.

To perform DDH analysis of *R. qingshengii* DSM 45222[T] and *R. jialingiae* DSM 45257[T] cells were disrupted by using a Constant Systems TS 0.75 (IUL Instruments) and the DNA in the crude lysate was purified by using the method of Marmur (1961). This was necessary since other, less labour-intensive methods failed to purify suitable amounts of high-quality DNA samples for DDH analysis even from 7–8 g wet biomass. Moreover, biomasses used for the analysis were stored at −80 °C until DNA purification, since the preservation in 1 : 1 (v/v) 2-propanol/water mixture proved to be insufficient to prevent degradation of cells and nucleic acids prior to DNA purification. DDH was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer with a Peltier-thermostat-equipped 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

Results of the PCR amplification of the 16S rRNA genes with primers 27f and 1525r were surprising, since three environmental isolates (strains K5, RGN4 and Ba49) along with the type strain of *R. jialingiae* yielded a non-specific, approximately 1200 bp PCR product besides the approximately 1500 bp specific product, making direct sequencing impossible. Sequence analyses gave interesting results. The reported 0.2 % difference between 16S rRNA gene sequences of type strains of *R. qingshengii* and *R. jialingiae* was not found, because the variable sequence stretch upstream of the 1492r primer site does not exist. This observation was true for the environmental isolates as well. Consequently, all *R. qingshengii*–*R. jialingiae* strains investigated in this study share identical 16S rRNA sequences. The approximately 1200 bp long non-specific PCR products showed 97.7 % homology with the gene sequence of exoDNase V alpha chain of *R. erythropolis* PR4.

The *gyrB* gene was successfully amplified by PCR from all of the strains used in this study. Type strains of *R. qingshengii* and *R. jialingiae* shared 99.4 % *gyrB* gene homology, while they showed 98.6 % and 98.9 % homology with type strain of *R. erythropolis* (their closest relative based on the *gyrB* gene), respectively (Fig. 1a). The *gyrB*-based phylogenetic tree shows that *R. qingshengii*–*R. jialingiae* strains cluster close together and are clearly separated from other closely related species of the genus (Fig. 1a).

The *catA* gene was also detectable in all of the investigated strains. Surprisingly only one nucleotide difference was
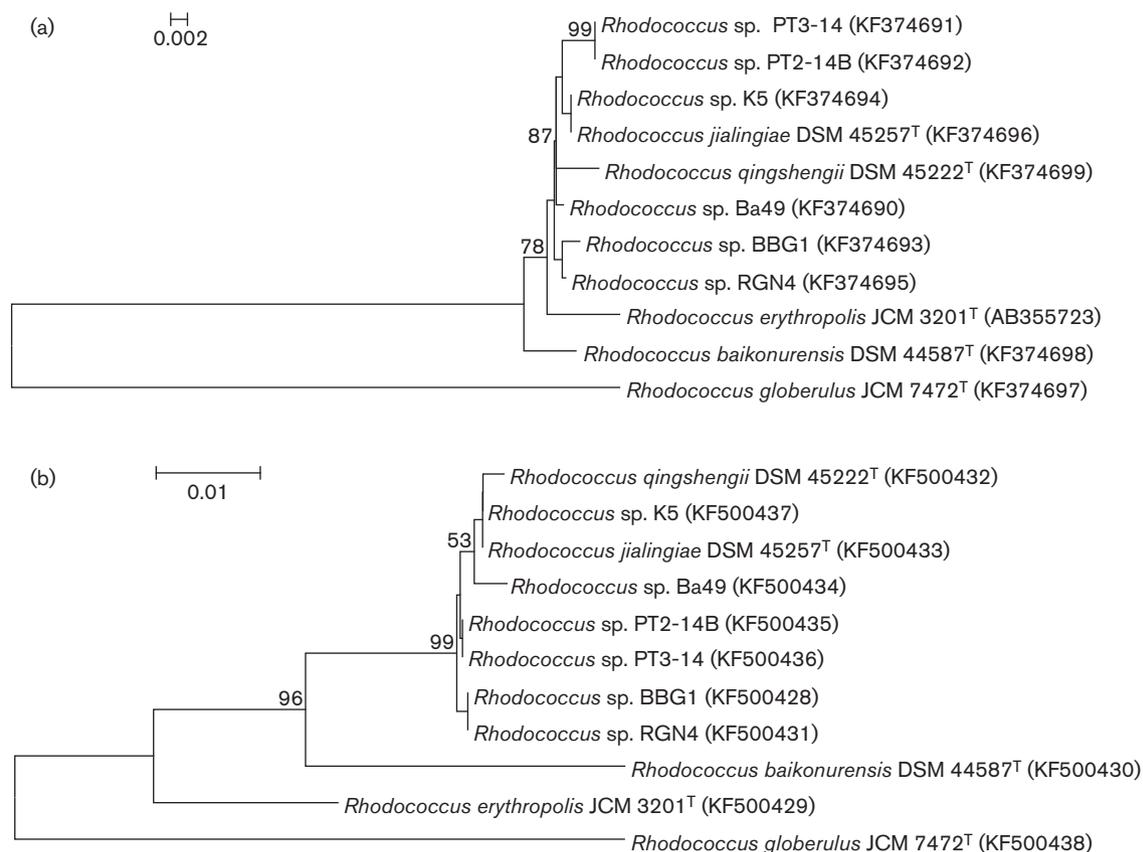
Fig. 1. Neighbour-joining trees based on (a) RHO-gyrBF/RHO-gyrBR amplified *gyrB* nucleotide sequences and (b) RHO-F/ RHO-R amplified *catA* nucleotide sequences showing the relationships between strains of the *Rhodococcus qingshengii–Rhodococcus. jialingiae* lineage and closely related species of the genus *Rhodococcus*. Nucleotide accession numbers are given in parentheses. Bootstrap values (%) of at least 50 % are shown at the branches. Bars, 0.002 and 0.01.

found between the *catA* nucleotide sequences of type strains of *R. qingshengii* and *R. jialingiae* on the studied sequence stretch. Consequently they shared 99.8 % *catA* gene homology, while they showed 95.2 % and 95.3 % homology with the type strain of *R. baikonurensis* (their closest relative based on the *catA* gene), respectively (Fig. 1b). The *catA*-based phylogenetic tree shows that all *R. qingshengii–R. jialingiae* strains cluster close together, while other species of the genus *Rhodococcus* are clearly separate from this branch (Fig. 1b).

These results were alarming, particularly in the light of the reported 27.7 % DDH value between type strains of *R. jialingiae* and *R. qingshengii* (Wang *et al.*, 2010). Such a low DDH value would presumably indicate larger differences in nucleotide sequences of functional genes. The lack of significant differences in the gene sequences investigated in the present study led us to reassess the DDH assay in case of these type strains. Our analysis resulted an 80.4 % DDH value, considerably over the cut-off-point recommended for the delineation of bacterial species (Wayne *et al.*, 1987).

Based on these results we concluded that the two type strains belonged to the same species.

Our results provide clear evidence against the current taxonomic status of *R. jialingiae* and *R. qingshengii* as two distinct species. Although Wang *et al.* (2010) demonstrated phenotypic differences between these type strains (e.g. colony colour, utilization of D-fructose, *myo*-inositol, D-mannitol and D-mannose) they should be considered as members of a single species. According to Stackebrandt (2011), the level of genome sequence identity among two strains must be higher than 96 % to reach a DDH similarity value of higher than 70 %. This means that strains affiliated to the same species may show 4 % differences in their genome sequences, allowing significant differences in phenotypic properties. According to Rule 42 of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1992), if taxa of equal rank are unified, the oldest legitimate name or epithet should be retained for the new combination. In this case, the epithet *qingshengii* has nomenclatural priority over the epithet *jialingiae*.

Concluding all of the results of this study *R. jialingiae* should be considered a later synonym of *R. qingshengii*.

Acknowledgements

References

Benedek, T., Vajna, B., Táncsics, A., Márialigeti, K., Lányi, S. & Máthé, I. (2013). Remarkable impact of PAHs and TPHs on the richness and diversity of bacterial species in surface soils exposed to long-term hydrocarbon pollution. *World J Microbiol Biotechnol* **29**, 1989–2002.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Huss, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria. 1990 Revision.* Washington, D.C.: American Society for Microbiology.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.

Máthé, I., Benedek, T., Táncsics, A., Palatinszky, M., Lányi, S. & Márialigeti, K. (2012). Diversity, activity, antibiotic and heavy metal resistance of bacteria from petroleum hydrocarbon contaminated soils located in Harghita County (Romania). *Int Biodeterior Biodegradation* **73**, 41–49.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. (2011). Molecular taxonomic parameters. *Microbiol Aust* **32**, 59–61.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Táncsics, A., Szoboszlay, S., Kriszt, B., Kukolya, J., Baka, E., Márialigeti, K. & Révész, S. (2008). Applicability of the functional gene catechol 1,2-dioxygenase as a biomarker in the detection of BTEX-degrading *Rhodococcus* species. *J Appl Microbiol* **105**, 1026–1033.

Wang, Z., Xu, J., Li, Y., Wang, K., Wang, Y., Hong, Q., Li, W. J. & Li, S. P. (2010). *Rhodococcus jialingiae* sp. nov., an actinobacterium isolated from sludge of a carbendazim wastewater treatment facility. *Int J Syst Evol Microbiol* **60**, 378–381.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Xu, J. L., He, J., Wang, Z. C., Wang, K., Li, W. J., Tang, S. K. & Li, S. P. (2007). *Rhodococcus qingshengii* sp. nov., a carbendazim-degrading bacterium. *Int J Syst Evol Microbiol* **57**, 2754–2757.