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Rhodococcus indonesiensis sp. nov. a new member of the *Rhodococcus ruber* lineage isolated from sediment of a neutral hot spring and reclassification of *Rhodococcus electrodiphilus* (Ramaprasad *et al*. 2018) as a later heterotypic synonym of *Rhodococcus ruber* (Kruse 1896) Goodfellow and Alderson 1977 (Approved Lists 1980)

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Abstract

A polyphasic study was designed to determine the taxonomic status of isolate CSLK01-03^T, which was recovered from an Indonesian neutral hot spring and provisionally assigned to the genus *Rhodococcus*. The isolate was found to have chemotaxonomic, cultural and morphological properties typical of rhodococci. It has a rod–coccus lifecycle and grows from 10 to 39°C, from pH 6.5 to 8.0 and in the presence of 0–10% (w/v) sodium chloride. Whole-organism hydrolysates contain *meso*-diaminopimelic acid, arabinose and galactose, the predominant menaquinone is MK-8 (H_2), the polar lipid pattern consists of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol mannosides, phosphatidylmethylethanolamine and two unidentified components, it produces mycolic acids, and $C_{16:0}$ is the major fatty acid. Whole-genome analyses show that the isolate and *Rhodococcus electrodiphilus* LMG 29881^T (GenBank accession: JAULCK000000000) have genome sizes of 5.5 and 5.1 Mbp, respectively. These strains and *Rhodococcus aetherivorans* DSM 44752^T and *Rhodococcus ruber* DSM 43338^T form well-supported lineages in 16S rRNA and whole-genome trees that are close to sister lineages composed of the type strains of *Rhodococcus rhodochrous* and related *Rhodococcus* species. The isolate can be distinguished from its closest evolutionary neighbours using combinations of cultural and phenotypic features, and by low DNA–DNA hybridization values. Based on these data it is proposed that isolate CSLK01-03^T (=CCMM B1310^T=ICEBB-06^T=NCIMB 15214^T) be classified in the genus *Rhodococcus* as *Rhodococcus indonesiensis* sp. nov. The genomes of the isolate and its closest phylogenomic relatives are rich in biosynthetic gene clusters with the potential to synthesize new natural products, notably antibiotics. In addition, whole-genome-based taxonomy revealed that *Rhodococcus electrodiphilus* LMG 29881^T and *Rhodococcus ruber* DSM 43338^T belong to a single species. It is, therefore, proposed that *R. electrodiphilus* be recognized as a heterotypic synonym of *R. ruber*.

INTRODUCTION

The genus *Rhodococcus* Zopf [1] has had a long and tortuous history [2–4], albeit one that was markedly improved by the application of numerical phenetic [5, 6] and polyphasic taxonomic procedures [7, 8]. Even so, it was only with the introduction of 16S rRNA gene sequence analyses and, subsequently, whole-genome-based phylogenomic studies that the full extent

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Abbreviations: GGDC, genome-to-genome distance calculator; RAxML, randomized axelerated maximum likelihood.

The DDBJ/ENA/GenBank accession numbers for the 16S rRNA gene and genome sequences of strain CSLK01-03^T are MK503551.1 and JAUBOF000000000, respectively.

Two supplementary figures and four supplementary tables are available with the online version of this article.

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of the heterogeneity within the genus was realized [9–11]. Sangal and his colleagues assigned representative rhodococci to multiple well-circumscribed species groups, as exemplified by *Rhodococcus equi* [6, 12], *Rhodococcus erythropolis* [6, 13], *Rhodococcus fascians* [14, 15] and *Rhodococcus rhodochrous* [1, 5] which encompassed two lineages, B1 and B2, the latter included the type strain of *Rhodococcus ruber* [6, 16]. Nouioui *et al.* [11] emended the description of these and other rhodococcal species and also transferred *Rhodococcus kunmingensis* [17] to the new genus *Aldersonia* as *Aldersonia kunmingensis*. Subsequently, *Rhodococcus cavernicola* [18] was assigned to the new genus *Spelaeibacter* as *Spelaeibacter caverinola* [19], while the genus *Prescotella* provided a stable home for *R. equi* and four closely related *Rhodococcus* species [20]. Like *Rhodococcus*, all of these new genera belong to the family *Nocardiaceae* [21, 22]. Genome-based analyses have also shown that several *Rhodococcus* species are later heterotypic synonyms of previously described species of *Rhodococcus* [18, 23].

Given the changes outlined above, the genus *Rhodococcus* contains around 50 validly named species (https://lpsn.dsmz.de/genus/rhodococcus) including *R. rhodochrous*, the type species, and the recently described *Rhodococcus oxybenzonivorans* [24], *Rhodococcus pseudokoreansis* [25], *Rhodococcus spelaei* [23] and *Rhodococcus spongicola* [26]. The revised genus encompasses aerobic, Gram-stain-positive to Gram-stain-variable, nonmotile, partially acid-fast actinomycetes with a rod–coccus growth cycle. Whole-organism hydrolysates are rich in *meso*-2.6-diaminopimelic acid, arabinose and galactose, muramic acid moieties are *N*-glycolated, the predominant menaquinone is MK-8 (H_2), the major phospholipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol mannosides, mycolic acids are present, and fatty acids consist of major proportions of straight-chain saturated, monounsaturated and 10-methyl branched chain components [23–26]. Draft genome sizes range from 3.9 to 10.4 Mbp and DNA G+C contents from 61.8 to 70.7 mol% [11, 13, 26]. Rhodococci are common in diverse natural habitats [25, 27], and are a source of valuable bioactive compounds, such as antimicrobial agents, bioflocculants, biosurfactants and enzymes [28–30]. Some are pathogens of animals, including humans [27, 31] and others are serious plant pathogens [32].

The improved classification of the genus *Rhodococcus* provides a sound framework for the discovery of novel rhodococci of potential biotechnological value. This study, a continuation of our earlier work on the diversity and bioactivity of filamentous actinomycetes from Indonesian extreme habitats, was designed to establish the taxonomic status of a presumptive *Rhodococcus* strain isolated from a neutral hot spring. Isolate CSLK01-03^T was compared with the type strains of closely related *Rhodococcus* species in a polyphasic taxonomic study. In addition, the draft genomes of the isolate and its closest phylogenomic neighbours were checked for biosynthetic gene clusters (BGCs) predicted to express for specialized secondary metabolites The isolate was found to belong to a new *Rhodococcus* species, *Rhodococcus indonesiensis* sp. nov., within the *R. ruber* lineage. The draft genomes of isolate CSLK01-03^T and its closest neighbours are rich in BGCs with the potential to synthesize new natural products.

ISOLATION, MAINTENANCE AND CULTIVATION

Strain CSLK01-03^T was isolated from a gravelly, non-saline neutral hot spring sediment (pH 6.7±0.4., organic matter 0.01±0.1 %) collected from the chimney of the Cisolok geyser (6° 56′ 1.199″ S 106° 27′ 12.402″ E) in the Sukabumi district, West Java Province, Indonesia, on 8 September 2016). Aliquots (100 ul) of a 10^{-1} sediment sample in ¼ strength Ringer's solution (Oxoid) were spread over three plates of actinomycete isolation agar (HiMedia), pH 7.3, which were incubated at 37 °C for 7 days. The isolation plates had been dried at room temperature for 30 min before inoculation, as recommended by Vickers and Williams [33]. A distinctive red colony growing on one of the selective isolation plates was used to inoculate plates of yeast extract–malt extract agar [International *Streptomyces* Project (ISP) medium 2] [34], which were incubated at 37 °C for 7 days. Biomass for the chemotaxonomic analyses was harvested from ISP2 broth cultures which had been shaken at 363 *g* for 14 days at 28 °C. The harvested biomass was washed twice in sterile distilled water and freeze-dried. Working cultures of the isolate, *Rhodococcus aetherivorans* DSM 44752^T [8], *Rhodococcus electrodiphilus* LMG 29881^T [35] and *R. ruber* DSM 43338^T were maintained on ISP2 agar plates., the strains were kept as mixtures of rods and cocci in 20%, v/v glycerol at −20 °C and at −80 °C for long-term preservation.

ACQUISITION OF CHEMOTAXONOMIC, CULTURAL AND MORPHOLOGICAL PROPERTIES

The isolate was examined for phenotypic properties known to be characteristic of *Rhodococcus* strains [18, 25, 27]. Colony morphological properties were recorded from an ISP2 agar plate [34] after incubation for 7 days at 28 °C and motility checked in ISP2 broth supplemented with 0.4% (w/v) agar. Smears prepared from growth on ISP3 agar were examined by light microscopy following Gram and Ziehl-Neelsen staining. Growth from the ISP2 agar plate incubated at 28 °C for 7 days was examined using a scanning electron microscope (Tescan Vega 3, LMU instrument), as described by O'Donnell *et al.* [36]. In addition, the cellular morphology of the isolate was examined after growth for 2, 4 and 7 days on IPS3 agar at 28 °C using a light microscope following Gram-staining. Standard chromatographic techniques were used to determine the isomers of diaminopimelic acid (A_2pm) [37], whole-organism sugars [38], mycolic acids [39], and polar lipids [40]. Menaquinones were extracted following standard procedures and revealed using HPLC, as described by Minnikin *et al.* [40]. The acyl group of the

muramic acid of the peptidoglycan was determined using the method of Uchida *et al.* [41]. Cellular fatty acids were extracted from freeze-dried cells of the isolate and fatty acid methyl esters (FAMEs) prepared following saponification and methylation using the procedure introduced by Miller [42] and modified by Kuykendall *et al.* [43]. The FAMEs were separated by gas chromatography (Agilent 68908) and the resultant peaks integrated automatically. The identity of the fatty acid names were determined using the standard Microbial Identification (MIDI) system, version 4.5 and the ACTIN 6 database [44].

The isolate exhibited chemotaxonomic, cultural and micromorphological properties typical of *Rhodococcus* strains [18, 20, 23, 24, 27]. The organism proved to be an aerobic, Gram-stain-positive, partially acid-alcohol-fast, nonmotile actinomycete which was shown to form rod- and coccoid-like elements and orange red colonies on ISP3 agar (Fig. S1, available in the online version of this article) after 7 days, and a rod–coccus lifecycle following growth on ISP3 for 2, 4 and 7 days. The major isoprenologues were MK-8 (23.2%) and MK-8(H_2) (76.2%) and the polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides with unidentified glyco- and phospho-lipids (Fig. S2). The cell-wall diamino acid was *meso*-A_{2pm}, the acyl type of muramic acid was *N*-gylcolated, and whole-cell hydrolysates contained arabinose, galactose and traces of glucose and ribose. The isolate also contained mycolic acids which had the same Rf +value as those extracted from the type strain of *R. rhodochrous*.

The fatty acids of the isolate consisted of major proportions (>10%) of $C_{16:0}$ (37.65%), $C_{18:0}$10-methyl-tuberculostearic acid (TBSA) (12.05%), $C_{18:1}$ ω9*c* (11.88%) and summed feature 5 ($C_{18:2}$ ω6/anteiso-$C_{18:0}$ 9*c*; 10.42%). The complete fatty acid profile is given in Table S1. The major fatty acids shared by the isolate and the three type strains are $C_{16:0}$, $C_{18:1}$ ω9*c*, $C_{18:0}$ 10-methyl tuberculostearic acid (TBSA) and summed feature 3.

More pronounced differences found between the polar lipid patterns of the type strains of these species, as reported by Ramaprasad *et al.* [35], were reflected in the polar lipid pattern of the isolate as it is the only one of these strains that contained phosphatydilmethylethanolamine and a glycolipid. The whole-organism hydrolysates of the isolate and *R. electrodiphilus* LMG 29881^T and other reference strains contained arabinose and galactose (diagnostic components), glucose and ribose [35].

CHARACTERIZATION OF GENOMES

Genomic DNA was extracted from wet biomass of single, well-separated colonies of the isolate and *R. electrodiphilus* LMG 29881^T grown on ISP2 agar for 7 days at 28°C using the protocol provided by MicrobesNG (Birmingham, UK; www.microbesng.uk) and sequenced on an MiSeq instrument (Illumina). The quality of the extracted DNA samples and sequencing of the genomic DNA libraries was achieved, as described by Kusuma *et al.* [45]. The libraries were sequenced following the 2×250 bp paired-end protocol (MicrobesNG), reads under 200 bp were discarded and contigs assembled using SPAdes software version 3.1.1 [46]. The draft genome assemblies of the strains were annotated using the RAST-SEED webserver [47, 48] with default options and are available from GenBank. The genome size of the isolate (5.5 Mbp) is smaller than that of *R. aetherivorans* DSM 44752^T (6.4 Mbp) but higher than those of the type strains of *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T(5.1–5.3 Mbp, respectively), the digital G+C similarities of the strains were within a 1 mol% G+C range (Table S2).

PHYLOGENY AND COMPARATIVE GENOMICS

An almost-complete 16S rRNA gene sequence (1523 nt; GenBank accession number MK503551.1) was retrieved directly from the draft genome of the isolate using the ContEst16S tool from the EzBioCloud webserver (www.ezbiocloud.net/tools/contest16s) [49]. To check the sequence authenticity, this had been compared with the associated 16S rRNA gene sequence generated using Sanger method following pairwise alignment. The resultant 16S rRNA gene sequence was aligned with corresponding ones of representatives of *Rhodococcus* species downloaded from the EzBioCloud webserver [50] using MUSCLE software [51]. Pairwise sequence similarities were assessed using the single-gene tree option from the Genome-to-Genome Distance Calculator (GGDC) webserver [52, 53]. Phylogenetic trees were inferred using the maximum-likelihood (ML) [54], maximum-parsimony [55] and neighbour-joining [56] algorithms using MEGA X software [57]. The robustness of the clades in the phylogenetic trees were determined using bootstrap analyses based on 1000 replicates [58]. The ML tree was rooted with *Nocardia asteroides* DSM 43757^T.

The assignment of *R. rhodochrous* and *R. ruber* clades to closely related lineages in the *Rhodococus* 16S rRNA gene tree (Fig. 1) is in good agreement with earlier studies [10, 27, 30, 35] though in other rhodococcal trees members of these taxa were assigned to distinct subclades [3, 19, 23]. The phylogenetic tree shows that isolate CSLK01-03^T belongs to the *R. ruber* lineage including *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T, sharing sequence similarities with these species of 99.0, 99.2 and 99.2%, respectively (Table S3). These strains are well separated from the *R. rhodochrous* clade.

The draft genome sequences generated from the isolate and *R. electrodiphilus* LMG29881^T were compared with the corresponding ones of their closest phylogenetic neighbours retrieved from the NCBI genome database using the codon tree option in the PATRIC website [59, 60], as described by Kusuma *et al.* [61], and an ML phylogenetic tree reconstructed with the RAxML algorithm [62]. The completeness and potential contamination in the draft genomes were checked using the CheckM platform [63] as shown in

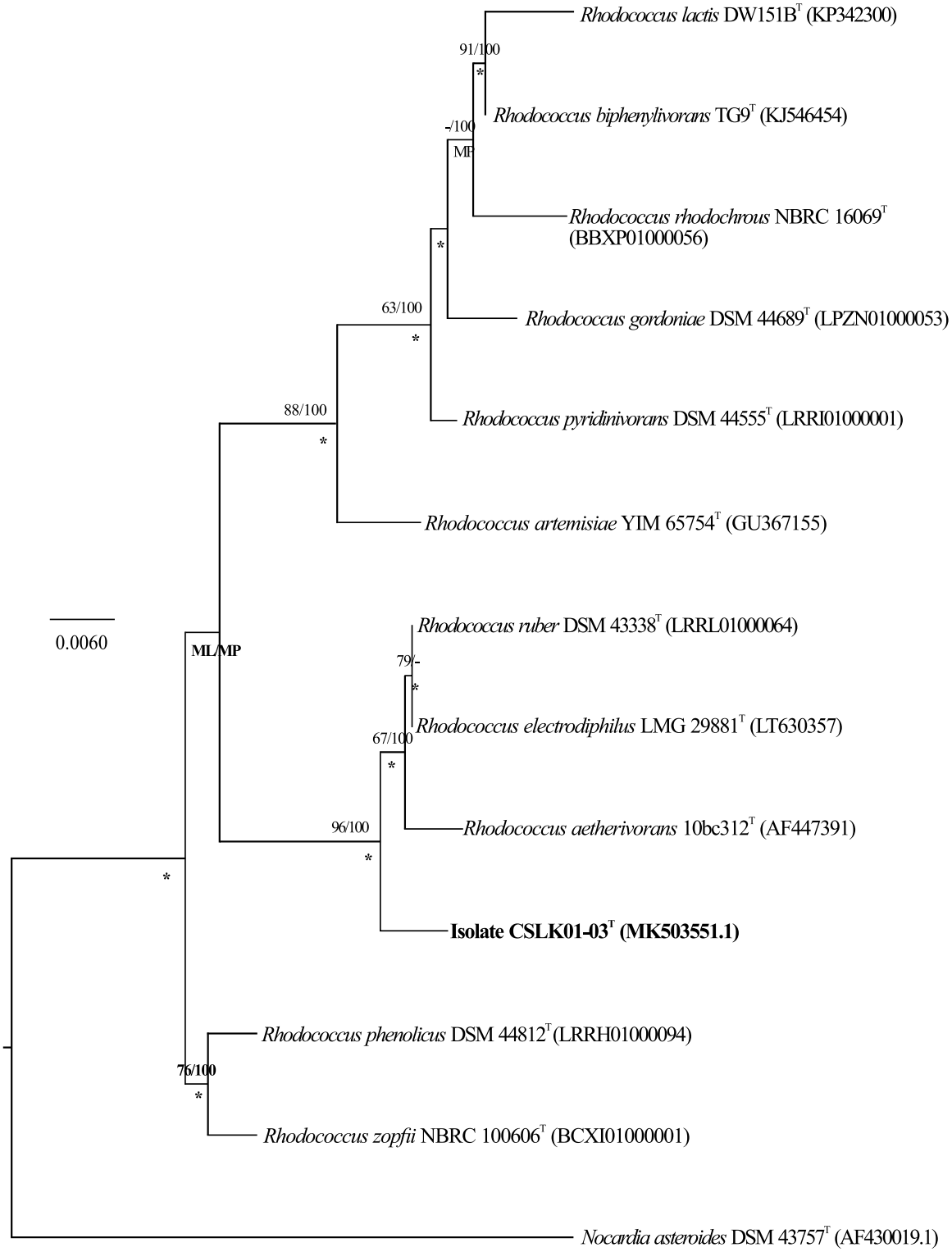

Fig. 1. Maximum-likelihood tree inferred using the GTR+GAMMA model based on almost complete 16S rRNA gene sequences showing relationships between isolate CSLK01-03^{T} and the type strains of closely related *Rhodococcus* species. Numbers above the nodes indicate bootstrap support values above 60% for the maximum-likelihood (left) and maximum-parsimony (right) algorithms. Asterisks indicate branches recovered using the neighbour-joining algorithm. GenBank accession numbers are shown in parentheses. The tree is rooted using the type strain of *Nocardia asteroides*, the type strain of the type species of the genus *Nocardia*, the type genus of the family Nocardiaceae.

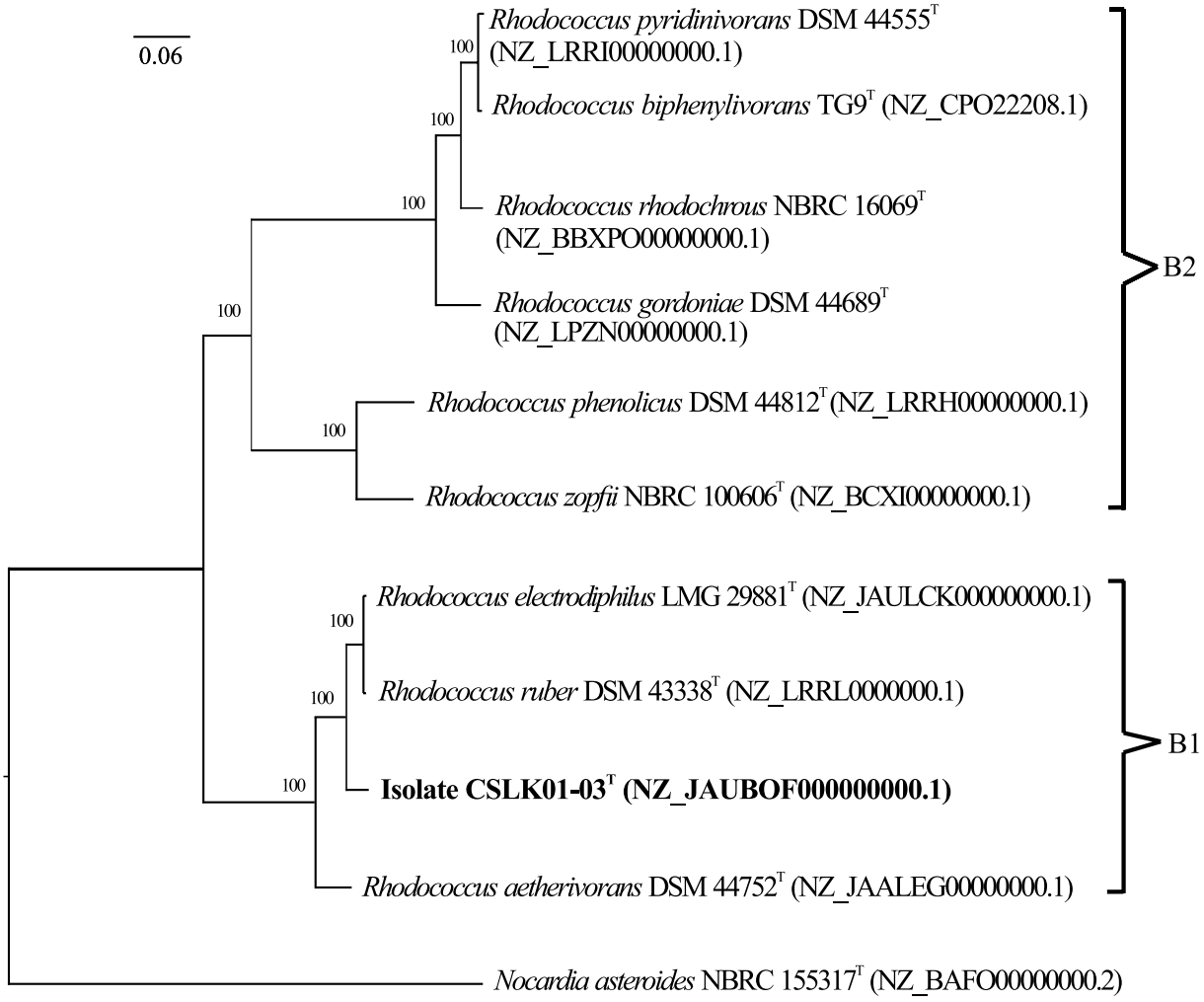

Fig. 2. Maximum-likelihood phylogenomic tree showing relationships between isolate CSLK01-03^T and the type strains of closest related *Rhodococcus* species. Numbers at the nodes are bootstrap support values based on 100 replicates. GeneBank accession numbers are shown in parentheses. The scale bar indicates 0.06 substitutions per nucleotide position. The tree is rooted using the type strain of *Nocardia asteroides*, the type species of the genus *Nocardia*, the type genus of the family Nocardiaceae.

Table S2. Fig. 2 shows that the strains assigned to species groups B1 and B2 [9, 10] belong to sister lineages. It is also evident that isolate CSLK01-03^T is most closely related to *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T, an association supported by a 100% bootstrap value; *R. aetherivorans* DSM 44752^T is closely associated with this taxon.

Orthologous average nucleotide identity (orthoANIu [64]) and digital DNA–DNA hybridization (dDDH [52]) values were determined between the isolate and its closest phylogenomic neighbours using the ANI calculator from the EzBioCloud (www.ezbiocloud.net/tools/ani) and GGDC (https://ggdc.dsmz.de/) webservers, and recommended thresholds used to define species boundaries, namely 95–96% [65, 66] and 70% [52, 53], respectively. Table 1 shows that the dDDH values between the isolate and its closest phylogenomic neighbours are well below the recommended cut-off point while the corresponding orthoANIu values are below the 96% threshold. These metrics show that the isolate is most closely related to the *R. electrodiphilus* LMG 29881^T; these results are consistent with the assignment of the isolate to a novel *Rhodococcus* species. It is interesting that the orthoANIu and DDH values between *R. ruber* DSM 43338^T and *R. electrodiphilus* LMG 29881^T are 99.4 and 94.1%, respectively, indicating that these strains belong to the same species.

PHENOTYPIC TRAITS

Isolate CSLK01-03^T, *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T were examined for phenotypic properties known to be of value in rhodococcal systematics [23–25]. Biochemical, degradation and physiological

Table 1. Average nucleotide identity (orthoANIu) and digital DNA–DNA hybridization (dDDH) values found between isolate CSLK01-03^T and the type strains of its closest phylogenomic neighbours

ANI/dDDH values (%)	Isolate CSLK01-03^T	*Rhodococcus electrodiphillus* LMG 29881^T	*Rhodococcus ruber* DSM 43338^T	*Rhodococcus aetherivorans* DSM 44752^T
Isolate CSLK01-03^T	100/100	95.4/61.5	95/60.4	91.3/43.7
Rhodococcus electrodiphillus LMG 29881^T	95.4/61.5	100/100	99.4/94.1	91.5/44.9
Rhodococcus ruber DSM 43338^T	95/60.4	99.4/94.1	100/100	91.4/44.7
Rhodococcus aetherivorans DSM 44752^T	91.3/43.7	91.5/44.9	91.4/44.7	100/100

properties were acquired using media and methods described by Williams *et al.* [67] and enzymatic profiles using API-ZYM strips (bioMérieux). The ability of the strains to grow under different temperature and pH regimes and in the presence of various concentrations of sodium chloride were recorded using ISP2 agar as the basal medium; pH values were adjusted using phosphate buffers. All of the tests were carried out in duplicate using a standard inoculum corresponding to 5.0 on the McFarland scale [68]. Cultural and growth features of the strains were recorded from tryptone–yeast extract, yeast extract–malt extract, oatmeal, inorganic salts–starch, glycerol–asparagine, peptone–yeast extract–iron and tyrosine agar plates (ISP media 1–7) [34] after 21 days at 28°C. Colony colours were determined by comparison against colour charts [69].

The duplicated cultures gave identical results for all of the phenotypic tests. It is also encouraging that the results obtained from the biochemical, degradation and carbon source tests on *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T were in good agreement with corresponding results from previous studies [6, 8, 35]. Some of the phenotypic properties are common to all of the strains but other distinguish between them, as shown in Table 2. The novel isolate, unlike *R. electrodiphilus* LMG 29881^T, its closest phylogenetic neighbour, was positive for alkaline phosphatase, esterase (C4) and β-glucoronidase, and used maltose and sodium gluconate as sole carbon sources. Conversely, *R. electrodiphilus* LMG 29881^T was positive for α-chymotrypsin, α-fucosidase and cystine, leucine and valine arylamidases, degraded casein, guanine, starch and Tween 40, and used N-acetylglucosamine, cellulose, galactose, mannitol, L-rhamnose and sodium butyrate as sole carbon sources, grew at pH 11 and 40°C, and in the presence of 13%, w/v sodium chloride. Combinations of other phenotypic features separate the isolate and *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T. However, it is clear that the R. *electrodiphilus* and *R. ruber* strains are closely related as they have 50 out of 72 phenotypic properties in common (71%). All of the strains can be distinguished by the extent to which they grow and formed pigments on ISP media (Table S4).

BCGS PREDICTED TO ENCODE FOR SPECIALIZED METABOLITES

Bioclusters in the draft genomes of the isolate and its closest phylogenomic neighbours were detected using the AntiSMASH 5.0 webserver (https://antismash.secondarymetabolites.org) [70] following a default setting with the cluster finder option off. Selected BGCs found in the genomes of the strains were compared with publicly available BGCs sequences held in the Minimum Information about Biosynthetic Gene Cluster Database, version 2 (MIBiG 2.0) [71] to distinguish novel from known bioclusters. Similarly, version 2 of the Antibiotic Resistance Target Seeker (ARTS 2.0) platform [72] was used to find bioclusters in the genome of the isolate predicted to encode for uncharacterized bioclusters based on the presence of resistant target genes, as described by Alanjary *et al.* [73]. In addition, the NCBI GenBank annotation pipeline and RAST were used to detect genes potentially associated with the ability of the isolate CSLK01-03^T to grow in the neutral hot spring.

The draft genomes of isolate CSLK01-03^T and its closest phylogenomic neighbours contained between 26 and 37 BGCs, as shown in Fig. 3. The genome of all of the strains harboured bioclusters predicted to encode for ectoines and heterobactins ('core' specialized metabolites). The latter were the first characterized siderophores isolated from rhodococci [74]. Seventy-four out of the 121 bioclusters (61%) were predicted to encode for unknown specialized metabolites, notably ones associated with non-ribosomal peptide synthases, others were predicted to express for known antibiotic compounds, such as icosalides A-B (100% gene sequence similarity), mycosubtilin (30%) and skyllamycin (4%). Detailed AntiSMASH results obtained for isolate CSLK01-03^T and for its closest phylogenomic neighbours are available online at (https://bit.ly/489SEkD).

Most of the bioclusters were either strain specific or present in more than one of the genomes. The draft genomes of the isolate and *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T, for instance, contained bioclusters predicted to encode for reveromycin A, an anti-tumour compound [75], methylenomycin, a broad-spectrum antibiotic [76], telomycin, an antibiotic which inhibits Gram-positive bacteria [77], and a cyclic peptide RP-1776, an anti-platelet-derived growth factor (PDGF) [78], respectively. The genomes of the isolate and *R. electrodiphilus* LMG 29881^T contained a biocluster predicted to encode for lympostin, a novel immunosuppressant from *Streptomyces* sp. KY11783 [79].

Table 2. Phenotypic characteristics which distinguish isolate CSLK01-03^T from the type strains of its closest phylogenomic neighbours

Strains: 1, CSLK01-03^T; 2, *Rhodococcus aetherivorans* DSM 44752^T; 3, *Rhodococcus electrodiphilus* LMG 29881^T; 4, *Rhodococcus ruber* DSM 43338^T. The strains were catalase positive, hydrolysed aesculin, arbutin and gelatin, reduced nitrate, degraded adenine (0.5%, w/v), hypoxanthine (0.4) and uric acid (0.4) and used fructose, glucose, ribose, xylose, acetate, benzoate, pyruvate and succinate (sodium salts) as sole carbon sources. None of the strains were oxidase-positive, produced esterase lipase (C8), lipase (C14), α-galactosidase, α-galactosidase, α-mannosidase or trypsin (API-ZYM tests), degraded elastin (0.3), keratin (1), tributyrin (0.1), Tween 20 (1) or xanthine (0.4) or used L-arabinose, arbutin or trehalose as sole carbon sources. They all grew optimally between pH 6.5–7.5, at 28–37 °C and in the presence of 1–2% sodium chloride. +, Positive; –, negative.

Characteristic	1	2	3	4
Enzyme activities (API ZYM):				
Acid phosphatase, β-galactosidase, α-glucosidase, naphthol-AS-BI-phosphohydrolase	+	–	+	+
Alkaline phosphatase	+	–	–	+
α-Chymotrypsin, α-fucosidase	–	–	+	–
Cystine, leucine and valine arylamidases	–	+	+	–
Esterase (C4), β-glucoronidase	+	–	–	–
N-Acetyl-*β*-glucosaminidase	–	–	–	+
β-Glucosidase	+	+	+	–
Biochemical tests:				
Allantoin hydrolysis	+	–	+	–
Urea hydrolysis	+	–	–	+
Degradation tests (% w/v):				
Casein (1)	–	+	+	–
Chitin (0.4)	+	–	+	–
Guanine (0.3), Tweens 60 and 80 (1)	–	–	+	+
Starch (0.1), Tween 40 (1)	–	+	+	+
Xylan (0.4)	+	+	+	–
Growth on sole carbon sources (1%, w/v):				
N-Acetylglucosamine, D-galactose, D-mannitol, sucrose	–	+	+	–
Cellulose, L-rhamnose	–	–	+	–
Cellobiose, *myo*-inositol	+	–	+	–
Maltose	+	+	–	+
D-Sorbitol	+	–	+	+
Sodium butyrate	–	–	+	+
Sodium gluconate	+	–	–	+
Tolerance tests:				
Growth in the presence of 13%, w/v sodium chloride	–	–	+	–
Growth at pH 11	–	–	+	–
Growth at 10°C	+	–	+	+
Growth at 40°C	–	+	+	+

Similarly, a biocluster predicted to express for rhodochelin, a unique mixed type of catechoalate hydroxamate siderophore synthesized by *Rhodococcus jostii* RHA1 [80] was only found in the genomes of *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T. The genomes of all of the strains, apart from *R. electrodiphilus* LMG 29881^T, contained a BGC associated with the synthesis of echosides, *para*-terphenyl compounds which inhibit DNA topoisomerase I and IIα, the latter were first isolated from *Streptomyces* sp. LZ35 [81].

The ARTS analyses on the draft genome of the isolate highlighted two bioclusters, BGC 5.1 and BGC 37.1, which are predicted to express for angucycline sch-47554/sch-47555 (3% gene sequence similarity) and atratumycin-like (10%) antibiotics, respectively. The former is an anti-fungal compound that was initially detected in the culture filtrate of *Streptomyces* strain

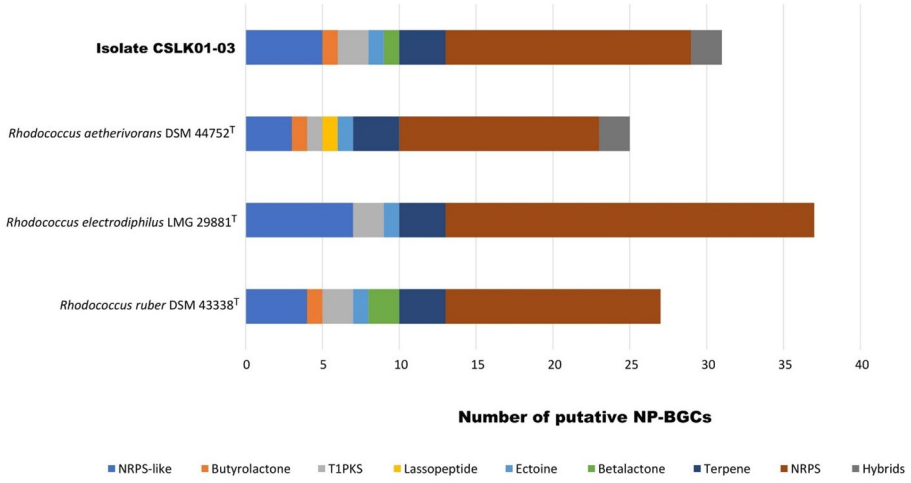

Fig. 3. Putative natural product biosynthetic gene clusters detected in the draft genome of isolate CSLK01-03^T and those of the type strains of closely related members of the *R. rhodochrous* species-group using AntiSMASH 5.0.

SCC-2136 isolated from a soil sample collected in Edmonton, Alberta, Canada [82] and the latter, a cyclodepsipeptide molecule known for its anti-mycobacterial properties, was first detected from *Streptomyces atratus* strain SCSIO ZH16NS-80S recovered from a deep-sea sediment [83, 84].

The whole-genome analyses of isolate CSLK01-03^T showed the presence of 101 putative genes considered to be associated with stress responses and environmental conditions that prevail in neutral hot springs, as exemplified by those encoding for DNA repair mechanisms (71 genes), oxidative stress (30 genes), osmoregulation (one gene) and heat shock response (one gene). In addition, the genome of the isolate included genes associated with resistance towards elevated heavy metal concentrations (notably, cobalt, zinc and cadmium; nine genes), and ones maintaining copper homeostasis (11 genes). The comprehensive results of the whole-genome analyses are provided at (https://bit.ly/3LirAGn).

The results outlined above provide further evidence that rhodococci isolated from extreme environments can be seen as attractive candidates for natural product discovery programmes, especially given their relatively fast rate of growth and availability of genetic tools which can be used for genetic modification [28].

TAXONOMIC CONCLUSIONS

It can be concluded from the genomic, phylogenetic and phylogenomic data that the isolate is a member of the *R. ruber* lineage, an integral component of the *R. rhodochorus* species-group [11, 12]. It can be distinguished from *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T and *R. ruber* DSM 43338^T, its closest phylogenomic neighbours, using a combination of phenotypic properties, and critically by low dDDH and OrthoANI values. It is, therefore, proposed that isolate CSLK01-03^T (CCMM B1310^T=ICEBB-06^T=NCIMB 15214^T) be recognized as the type and only strain belonging to *Rhodococcus indonesiensis* sp. nov. It is also clear from the evolutionary trees and associated phenotypic data that the type strains of *R. electrodiphilus* and *R. ruber* are very closely related. Critically, these relationships are underpinned by ANI and dDDH similarities that are well above the respective cut-off points used to assign closely related strains to the same species. Consequently, it is proposed that *R. electrodiphilus* [35] be classified as a later heterotypic synonym of *R. ruber* [6, 16].

DESCRIPTION OF *RHODOCOCCUS INDONESIENSIS* SP. NOV.

Rhodococcus indonesiensis (in.do.ne.si.en'sis N.L. masc. adj. *indonesiensis*, of or pertaining to Indonesia, the source of the isolate).

Aerobic, Gram-stain-positive, partially acid-alcohol fast, non-motile actinomycete which forms short rods (0.8–1.5 µm) and coccoid-like elements (0.9–1.1 µm). Smooth, convex, reddish orange colonies with glistening surfaces are formed on oatmeal agar. Grows well on ISP media 2, 3 and 6, from 10 to 37°C, optimally at 28°C, from pH 6.5 to 8.0, optimally around pH 7.0, and in the presence of 0–10%, w/v sodium chloride, optimally with 1%, w/v. Catalase-positive, oxidase-negative, reduces nitrate, hydrolyses aesculin, allantoin, arbutin, gelatin and urea, produces acid and alkaline phosphatases, esterase (C4), β-galactosidase, β-glucoronidase, α- and β-glucosidases, and naphthol-AS-BI-phosphohydrolase, but not α-chymotrypsin, α-fucosidase, esterase lipase (C8), lipase (C14), α-galactosidase, cystine arylamidase, α-galactosidase, leucine arylamidase,

α-mannosidase, trypsin or valine arylamidase (API-ZYM tests). Degrades adenine, chitin, hypoxanthine, uric acid and xylan, but not casein, elastin, guanine, keratin, starch, tributyrin, Tweens 20, 60 and 80, or xanthine. Cellobiose, D-fructose, D-glucose, *myo*-inositol, maltose, D-ribose, D-sorbitol, D-xylose, acetate, benzoate, gluconate, pyruvate and succinate (sodium salts) are used as sole carbon sources, but not *N*-acetylglucosamine, L-arabinose, arbutin, cellulose, D-galactose, D-mannitol, L-rhamnose, sucrose, trehalose or sodium butyrate. Whole-organism hydrolysates contain *meso*-$A_{2pm,}$ arabinose and galactose, muramic acid moieties are *N*-glycolated, the major cellular fatty acids are $C_{16:0}$, $C_{18:1}$ ω9*c*, $C_{18:0\text{-}10\text{-}}$methyl-tuberculostearic acid (TBSA) and summed feature 5 ($C_{18:2}$ ω6/anteiso-$C_{18:0}$ 9*c*), the predominant menaquinone is MK-8 (H_2), and the diagnostic polar lipid is phosphatidylethanolamine. Produces mycolic acids. The genome of the type strain is 5.5 Mbp and the DNA G+C content is 70.1 mol%.

The type strain, CSLK01-03^T (CCMM B1310^T=ICEBB-06^T=NCIMB 15214^T), was isolated from a gravelly, neutral hotspring sediment taken from the chimney of the Cisolok geyser, Sukabumi District, West Java Province, Indonesia. The GenBank accession number of the type strain is JAUBOF000000000.

EMENDED DESCRIPTION OF *RHODOCOCCUS RUBER* [6, 16] (APPROVED LISTS 1980)

Heterotypic synonym: *Rhodococcus electrodiphilus* Ramaprasad *et al.* 2018 [35] 2648^{AL}

Description is based on data from this and previous studies [6, 27, 35]

Aerobic, Gram-stain-positive, non-motile actinomycetes that form a branched substrate mycelium which fragments into rod- and coccoid-shaped cells. Grows from 10 to 40°C. Degrades adenine, guanine, hypoxanthine, starch, uric acid and Tweens 40, 60 and 80, but not elastin, keratin, tributyrin, Tween 20 or xanthine. Casein, arbutin and gelatin are hydrolysed and nitrate is reduced. Acid phosphatase, β-galactosidase, α-glucosidase and naphthol-AS-BI phosphohydrolase are produced but not esterase(C4), esterase lipase (C8), lipase (C14), α-galactosidase, α-mannosidase or trypsin. D-Fructose, D-glucose, D-ribose, D-sorbitol, D-xylose, acetate, benzoate, butyrate, pyruvate and succinate (sodium salts) are used as sole carbon sources, but not L-arabinose, arbutin or trehalose. Whole-organism hydrolysates are rich in *meso*-2,6-diamenopimelic acid, arabinose and galactose. The predominant menaquinone is MK-8(H2). Mycolic acids are present. Genome sizes range from 5.1 to 5.5 Mbp and the corresponding digital G+C contents from 70.4 to 70.7 mol%. The type strain DSM 43338^T was isolated from soil.

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Author contributions
M.G., I.N., A.B.K., G.F. and F.A. designed the study and prepared the manuscript. A.B.K., G.F. and F.A. helped to collect the sediment sample and characterized the isolate and associated strains under the supervision of M.G. and I.N. A.B.K. and G.F. deposited the type strain of the new species in the culture collections. All of the authors approved the final version of the manuscript.

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

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