

Rothia santali sp. nov., endophytic bacteria isolated from sandalwood (*Santalum album* L.) seedling

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Abstract

A novel, 'mustard yellow' pigmented aerobic bacterial strain designated AR01^T was isolated from hypocotyl tissue of a sandalwood seedling from Bangalore, India. The 16S rRNA gene of strain AR01^T had the highest 98.97% sequence similarity with *Rothia halotolerans* YIM 90716^T (KCTC 19172) followed by *Rothia kristinae* PM 129^T (NBRC 15354^T) (97.31%) and *Rothia koreensis* P31^T (JCM 15915) (97.11%), respectively. The strain AR01^T was coccoid-shaped, non-motile, non-spore-forming, oxidase-negative, and catalase-positive. The strain AR01^T has a genome size of 3.31 Mb containing 2993 protein-coding genes including 48 tRNA and 10 rRNAs spread across 84 contigs. The genomic DNA G + C content was 71.77 mol%. The calculated dDDH was 31.10%, and the OrthoANI value was 85.27% compared to its closest related type strain *Rothia halotolerans* YIM 90716^T. The predominant cellular fatty acids were C_{16:0 iso} (30.04%), C_{15:0 anteiso} (37.42%), and C_{17:0 anteiso} (21.78%). The strain AR01^T contains major polar lipids including diphosphatidylglycerol and phosphatidylglycerol. Based on the distinct physiological, biochemical characteristics and genotypic relatedness indicated that AR01^T represents a novel species of the genus *Rothia*, for which the name *Rothia santali* sp. nov. (Type strain AR01^T = MCC 4800^T = JCM 35593^T) is proposed.

The GenBank/EMBL/DDBJ accession number for the reference 16S rRNA gene sequences of the strain AR01^T is OM838448. The accession number of the whole-genome of AR01^T is JANAFB000000000

Introduction

The genus *Rothia* was initially classified as *Nocardia* (Onishi, 1949) which was later renamed *Rothia* by (Georg and Brown 1967). In the beginning, *Rothia* was a group under the *Actinomycetaceae* family which was then transferred to the *Micrococcaceae* family by Stackebrandt et al. 1997. They have described cells of *Rothia* are usually coccoid in morphology with raised smooth colonies, and gram-stain-positive. The cellular structure is mostly comprising peptidoglycan type A3α, and contains alanine, glutamic acid, and lysine. Accurate identification of *Rothia* by conventional method is tricky because of its high similarity to genus like *Kocuria* and *Nocardia*. In such cases, polyphasic approach of microbial taxonomical characterisation is employed for a more reliable and accurate approach for the novel identification of a species. To date, 15 *Rothia* species have been described; among which "*Rothia arfidiae*" (Ko et al. 2009), "*Rothia marina*" (Liu et al. 2013) and "*Rothia nasisuis*" (Schlattmann et al. 2018) are listed as 'invalidly published' species (Parte et al., 2020). Members of the genus *Rothia* have been mostly isolated from human skin and tooth, plant rhizosphere, saline soil, air, waste and estuarine water, and domesticated animals (Pandhi and Hammond 1975; Fotos et al. 1982; Bednář and Mára 1991; Collins et al., 2000; Chou et al., 2004; Fan et al., 2004; Li et al., 2004; Tang et al., 2009; Park et al. 2010; Xiong et al., 2013; Kämpfer et al., 2016; Ananieva et al. 2018; Nouioui et al. 2018). In this study, the strain AR01^T was isolated from hypocotyl tissue of 10–15 days old symptomatic sandalwood (*Santalum album* L.) seedlings which were screened for presence of pathogenic and commensal microbiome associated with high mortality rate during post germination period.

Materials And Methods

Isolation and Culture Conditions

The strain AR01^T was isolated from hypocotyl tissue of sandalwood seedlings showing symptoms of stunted and abnormal growth collected from insect-free sandalwood nursery at Indian Wood Science and Technology (IWST) institute located at Bangalore, India. The sandalwood seedling was surface sterilized by washing with Sodium Dodecyl Sulphate (L3771; Sigma Aldrich, Germany) and processed to obtain endophytic microbes. The pre-processed samples were further grown on R2A (M1743; HiMedia, India) and TSA (M1968; HiMedia, India) media, respectively. The plates were incubated at 28 °C for 24-48 hrs and checked for its growth. Morphologically different isolates from the mix, including new strain AR01^T, were further sub-cultured on LA (M557; HiMedia, India) medium till pure colonies were obtained. The purified strain AR01^T was maintained on LA plates at 4 °C, storage stock was prepared using 20 % (v/v) glycerol (MB060; HiMedia, India) and maintained at 80 °C, and in liquid nitrogen. The strain AR01^T was deposited to National Centre for Microbial Resource (NCMR), National Centre for Cell Science (NCCS), India with accession number MCC 4800 and Japan Collection of Microorganisms (JCM) with accession number JCM 35593.

Morphological, Physiological, and Biochemical Characteristics

The Gram-staining-nature of AR01^T was determined by observing the culture smear under a light microscope (Model BX53; Olympus, USA) with the help of a Gram staining kit (K001-KT; HiMedia, India). The size and shape of the cells were determined by scanning electron microscope (Carl Zeiss, EVO 18, Version 6.02). Prior to that the bacterial/ fungal samples were fixed in 2.5 % Glutaraldehyde solution (G5882; Merck, Germany) and dehydrated using graded alcohol concentrations of 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, and 90 %, one time and twice in 100% for 10 min each (Kannan, 2018). The dehydrated samples were coated with gold particles using a sputter coater (model, SC7620; Quorum technologies, UK).

For the media optimization, cells of strain AR01^T were grown in a different media plate including R2A, LA, NA, and TSA and incubated at 28 °C for 24 hr. Optimization of physiological characteristics like Temperature, pH, and salt tolerance was carried out in LB within a duration of 7 days each, respectively. Cell growth was tested at various temperatures, starting at 5 to 40 °C at an interval of 5 °C on LB medium. The cells' ability to sustain growth at different pH was checked at pH ranging from pH 4-12 units. Buffer systems were prepared as follows: acetate buffer (pH 4-5), phosphate Buffer (pH 6-7), and bicarbonate-carbonate buffer (pH 8-12). The salt tolerance of the culture was checked by inoculation in LB having the salinity range of 0 % to 5 % of NaCl with an increment of 0.5%, examined at 28 °C for 7 days.

The ability of AR01^T to produce spores was observed by Schaeffer–Fulton staining technique (Schaeffer–Fulton, 1933) with Methylene blue as the primary stain and 0.5 % Saffron as a secondary stain. Cell motility was checked by stabbing the culture in a motility test medium (M260; HiMedia, India).

The oxidase and catalase activity of the strain AR01^T was checked by oxidase discs (DD018; HiMedia, India) and 3 % (v/v) H₂O₂ (31642; Sigma Aldrich, Germany), respectively.

Utilization of carbon sources, assimilation and additional biochemical sensitivity tests were determined by performing API 20 NE (Biomerieux, France) and GEN III MicroPlate (BIOLOG, USA) assays according to standard manufacturer instructions. The enzyme activity against different substrates of strain AR01^T was performed using API ZYM (Biomerieux, France). The type strain of *R. halotolerans* YIM 90716^T (KCTC 19172) was used as a reference strain for all the tests mentioned earlier.

Chemotaxonomic Characterization

Cellular fatty acids of strain AR01^T were analyzed by Sherlock™ Microbial ID System (MIDI, Version 6.1; USA) using RTSBA6 library as per the manufacturer's instructions (Sasser 2001) using the cell mass of strain AR01^T and *R. halotolerans* YIM 90716^T (KCTC 19172) harvested by growing them on TSA media at pH 7.3±0.2 for 48 hr. For polar lipid analysis, cell mass was harvested from cultures at the logarithmic phase. Methanol/ chloroform/0.3 % sodium chloride (2:1:0.8, by vol.) was used for the extraction of polar lipids (Bligh and Dyer 1959) in addition to the modifications of (Card 1973). Two-dimension chromatography was used for separation using chloroform-methanol-water (65:25:4 by vol.) in the first dimension and chloroform-acetic acid-methanol-water (40:7.5:6:2, by vol.) in the second dimension on silica gel TLC (Kieselgel 60 F254; Merck) (Minnikin et al. 1984). The plates were dried and sprayed with 5 % ethanolic phosphomolybdic acid for visualization of total lipids. Further characterization was done by spraying the plates with ninhydrin (for amino groups), molybdenum blue (for phosphates), Dragendorff (for quaternary nitrogen), or α-naphthol (for sugars) (Card 1973).

Sequencing of the 16S rRNA gene and Phylogenetic Analysis

Genomic DNA of the strain AR01^T was extracted using the CTAB DNA extraction protocol (Ausubel, 1989). The quality and quantity were checked using a UV Spectrophotometer Nanodrop One, (Thermo Fisher Scientific, USA) and Qubit fluorometer (Thermo Fisher Scientific, USA). The variable region of the 16S rRNA gene was amplified using the bacterial universal primer 27F and 1492R. The purified PCR products were further sequenced using primers 343R, 704F, 907R, and 1028F (Baker et al., 2003). The obtained 16S rRNA gene sequence (OM838448, 1485 bp) of AR01^T was compared with the closest known sequences with valid names only according to EzBioCloud Database (Yoon et al. 2017). Phylogenetic trees were constructed using of Neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) methods to infer the position of the AR01^T and its closest known relatives using the MEGA7 (Kumar et al. 2016) software with 1000 replications of bootstrap analysis. The type strain *R. halotolerans* YIM 90716^T (KCTC 19172) was which was closest phylogenetic relative, procured from their respective culture collections for comparative polyphasic characterization and maintained on LA media at pH 7 and 28 °C.

Genome Sequencing and Analysis

Using Illumina MiSeq and Oxford Nanopore Technology (ONT) platform the genomic DNA of strain AR01^T was sequenced to obtain its genome sequence. The Illumina MiSeq raw reads were checked for their quality using FastQC v0.10.1 (Brown et al. 2017). The sequenced data obtained from ONT sequencing data were base-called with quality filtering (> Q7) using Guppy v3.5.4. The quality-filtered sequence of Illumina MiSeq and ONT reads were assembled using SPAdes version v3.15.3 (Antipov et al. 2016; Prjibelski et al. 2020) to obtain hybrid genome assembly of the strain AR01^T. The quality and completeness of the assembled genome were checked using QUAST v5.0.2 (Gurevich et al. 2013) and CheckM v1.1.3 (Parks et al. 2015). The rRNA and tRNA screening was done by running RNAmmer (version 1.2) as described by (Lagesen et al. 2007). The DNA G+C content of strain AR01^T were calculated. The digital DNA–DNA hybridization (dDDH) values were calculated using the Genome-to-Genome Distance Calculator (GGDC) webserver following the recommended formula 2 and orthoANI calculator for Average nucleotide identity (ANI), respectively (Meier-Kolthoff et al. 2014; Auch et al. 2010; Yoon et al. 2017). Further, the genome of strain AR01^T was annotated from the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Zhao et al. 2012). The functional groups of strain AR01^T were analyzed using Clusters of Orthologous Groups (COG) in eggNOG-mapper v2 (Cantalapiedra et al. 2021) from the PGAP annotated protein sequences. Comparison of orthologous gene clusters among the genomes under study was made using OrthoVenn2 (Xu et al. 2019).

To validate and confirm the taxonomic position of new strain AR01^T, a phylogenetic tree based on the core-genome was constructed using BPGA, version 1.3.0 (Chaudhari et al. 2016) and UBCG, version 3.0 (Na et al. 2018) of 10 type strains phylogenetically related to strain AR01^T. As described by Edgar 2010, using the integrated USEARCH algorithm the BPGA pipeline generated orthologous protein clusters. The generated protein sequences were further aligned and concatenated. Finally, the phylogenetic tree was reconstructed using the BPGA concatenated sequences by the neighbour-joining method in MEGA 7. A total of 18,883 amino acids position were constructed in the final phylogeny tree. In addition, UBCG identified the bacterial core gene of strain AR01^T and its closely related species. The identified genes were further concatenated, align and then processed for reconstructing the phylogenetic tree. The length of concatenated alignment was 93,480 bp containing 92 marker genes identified using HMMER (Potter et al. 2018) and predicted using Prodigal search (Hyatt et al. 2010).

Results And Discussion

Phylogenetic and Genotypic Analysis

The 16S rRNA gene of strain AR01^T (OM838448, 1485 bp) showed its highest sequence similarity to *R. halotolerans* YIM 90716^T (KCTC 19172) (Tang et al. 2009; Nouioui et al. 2018) with 98.97 % followed by *R. kristinae* PM 129^T (NBRC 15354) (97.31 %) (Kloos et al. 1974; Nouioui et al. 2018) and *R. koreensis* P31^T (JCM 15915) (Park et al. 2010; Nouioui et al. 2018) with 97.11 % similarity. Based on the 16S rRNA gene neighbor-joining phylogenetic tree construction of strain AR01^T formed a distinct monophyletic clade with *R. halotolerans* YIM 90716^T (KCTC 19172) (Figure 1).

The genome sequence of strain AR01^T generated 3,375,449 reads from the Illumina MiSeq and 3,399,524 reads from the ONT sequencing. Depth coverage of 80x was obtained with 99.01 % complete genome containing 84 scaffolds with the largest contigs of 2,20,631 bp. The DNA G+C contents were 71.77 mol %. The final genome assembly of strain AR01^T was deposited in NCBI GenBank bearing accession number JANAFB000000000. The digital DNA–DNA hybridization (dDDH) values for strain AR01^T in against *R. halotolerans* YIM 90716^T (KCTC 19172) were 31.10 % and 85.27% for orthoANI. A phylogenetic tree constructed from the core genome using BPGA and UBCG further supported the phylogeny derived using 16S rRNA gene sequences (Figure 2).

A total of 2,993 protein-coding genes were predicted including 48 tRNA and 10 rRNA. A total of 1,670 genes had specific functional distributions according to the COG categories. The functional genes were assigned to 18 functional categories and 1 category (458 genes) with unknown function. The genes responsible for Amino acid transport and metabolism (224 genes), transcription (199) and Translation, ribosomal structure and biogenesis (154 genes) were the most abundant followed by genes responsible for Inorganic ion transport and metabolism (139 genes), Energy production and conversion (136 genes) (Figure 3b). The Venn diagram represents 1415 gene clusters shared by AR01^T alone with its closely related type strains while 207 gene clusters shared by all strains (Figure 3a).

Morphological, Physiological and Biochemical Characteristics

Colonies of strain AR01^T were a mustard yellow pigmented colony, smooth surface with an entire margin, it is a non-endospore forming and non-motile. The cells of strain AR01^T were gram-positive, coccoid-shaped with 800 to 1000 nM in diameter in size as seen in scanning electron micrographs. Strain AR01^T showed an optimum growth in LA incubated at 25-30 °C (optimum 28 °C) for 2 days. The cells of AR01^T were able to grow on a salinity range of 0.0 % to 5.0 % at neutral pH of 7.0. However, the new strain was able to grow at a high pH of 10.0 (Table 1). Strain AR01^T was found to be catalase-negative and oxidase-positive.

Based on the API 20 NE test performed, strain AR01^T was found negative for nitrate reduction and could not hydrolyse ESCulin (β-glucosidase) whereas strain *R. halotolerans* YIM 90716^T (KCTC 19172) showed positive results. Assimilation of L-arabinose, D-mannose, N-acetyl-glucosamine, and potassium gluconate was detected negative in the strain KCTC 19172 but not in AR01^T. The APIZYM test performed showed a positive enzyme activity of alkaline phosphatase and α-glucosidase for strain AR01^T and negative for KCTC 19172. However, the esterase enzyme activity tested positive for strain KCTC 19172^T but not for strain AR01^T. In the BIOLOG GEN III microplate assays, strain AR01^T showed positive utilization of sugars such as D-Maltose, Stachyose, D-Raffinose, and D-Melibiose but not for the strain KCTC 19172^T. The strain KCTC 19172^T was found utilizing sugars such as β-Methyl-D-Glucoside, D-salicin, N-Acetyl-D-Glucosamine, N-Acetyl-β-D-Mannosamine, D-Galacturonic Acid, and L-Galactonic Acid Lactone as against the strain AR01^T. The strain AR01^T was found positive for utilising α-D-Glucose, D-Galactose, D-Fructose-6-PO₄, L-Arginine, L-Glutamic Acid, L-Serine, Lincomycin Guanidine HCl, Methyl Pyruvate and Propionic

acid whereas its closest strain KCTC 19172^T was found negative. Based on Biolog GEN III strain AR01^T was found resistant to Fusidic acid, D-serine, 1% sodium lactate, rifamycin SV, Vancomycin, Nalidixic Acid, Aztreonam, and Sodium Bromate whereas the comparing strain was tested negative. All data including the morphological, cultural, physiological and biochemical characteristics of strain AR01^T are given in table 1 and supplementary table S1.

Chemotaxonomic Characterization

The cellular major fatty acids contained in the strain AR01^T were C_{16:0 iso} (30.04 %), C_{15:0 anteiso} (37.42 %) and C_{17:0 anteiso} (21.78 %) (supplementary table S2). The major polar lipids present in strain AR01^T mainly comprised Phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) along with unidentified glycolipid (GL), traces of unidentified lipid 1-5 designated as L1, L2, L3, L4, L5, unidentified amino-phospholipid (APL) and unidentified phospholipid lipid (PL). The strain KCTC 19172^T contain fatty acid of Phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), unidentified lipid 1, 3, 5, unidentified glycolipid (GL), unidentified phospholipid lipid (PL) (Table 1).

Description of *Rothia santali* sp. nov.

Rothia santali (san.ta'li. L. masc. adj. *santali*, of the *Santalum*, isolated from host plant sandalwood, *Santalum album* L.)

Gram-staining-positive, coccoid-shaped, 800-1000 nm in size; non-endospore forming, and non-motile; catalase-negative and oxidase-positive; colonies mustard yellow-pigmented, smooth round with convex elevation (2-3mm in diameter) with the entire margin; grow at a temperature range of 25-30°C (optimum 28°C) at pH 7.0-10.0 with optimum pH of 7.0 and a salinity range of 0-5.0 % (w/v); alkaline phosphatase and α-glucosidase positive; positive assimilation of sugars like D-Maltose, Stachyose, D-Raffinose, D-Melibiose, α-D-Glucose, D-Galactose, D-Fructose-6-PO₄, L-Arginine, L-Glutamic Acid, L-Serine, Lincomycin Guanidine HCl, Methyl Pyruvate and Propionic acid; resistant to Fusidic acid, D-serine, 1% sodium lactate, rifamycin SV, Vancomycin, Nalidixic Acid, Aztreonam, and Sodium Bromate; contains polar lipids Phosphatidylglycerol and Diphosphatidylglycerol and cellular fatty acids C_{16:0 iso} (30.04%), C_{15:0 anteiso} (37.42%) and C_{17:0 anteiso} (21.78%).

The type strain AR01^T (= MCC 4800 = JCM35593) was isolated from hypocotyl tissues of sandalwood (*Santalum album* L.) seedlings located in Bangalore, India

Abbreviations

R2A	Reasoner's 2A
TSBA	Trypticase Soy Broth Agar
LA	Luria Agar

NA	Nutrient Agar
API	Analytical profile index
DPG	Diphosphatidylglycerol
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PL	Unknown phospholipid lipid
GL	Un-identified glycolipid
MEGA	Molecular Evolutionary Genetics Analysis
NJ	Neighbour-Joining
ML	Maximum Likelihood
MP	Maximum-Parsimony
UBCG	Up-to-date Bacterial Core Gene
PBGA	Bacterial Pan Genome Analysis
dDDH	Digital DNA–DNA hybridization
orthoANI	Orthologous average nucleotide identity

Declarations

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

No human or animal subjects were recruited for this study.

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Tables

Table 1

Differential phenotypic characteristics between *Rothia santali* AR01^T (MCC 4800), and its closest phylogenetic relative, *Rothia halotolerans* YIM90716^T (KCTC 19172).

Characteristics	AR01 ^T	KCTC 19172 ^T
Isolation source	Sandalwood	Saline soil
Optimum temperature range for growth (°C)	28	28
pH range for growth	pH 6–10	pH 6–8
Salt tolerance range for growth (%)	0–5	0–5
BIOLOG GEN III tests:		
D-Maltose	+	-
Stachyose	+	-
β-Methyl-D-Glucoside	-	+
D-Raffinose	+	-
D-Salicin	-	+
D-Melibiose	+	-
N-Acetyl-D-Glucosamine	-	+
N-Acetyl-β-D-Mannosamine	-	+
α-D-Glucose	+	-
D-Galactose	+	-
D-Fructose-6-P04	+	-
L-Arginine	+	-
L-Glutamic Acid	+	-
L-Serine	+	-
Lincomycin	+	-
Guanidine HCl	+	-
D-Galacturonic Acid	-	+
L-Galactonic Acid Lactone	-	+
Methyl Pyruvate	+	-
Propionic Acid	+	-

Characteristics	AR01 ^T	KCTC 19172 ^T
Chemical sensitivity assay:		
1% Sodium Lactate	+	-
Fusidic Acid	+	-
D-Serine	+	-
Rifamycin SV	+	-
Vancomycin	+	-
Nalidixic Acid	+	-
Aztreonam	+	-
Sodium Bromate	+	-
API 20 NE:		
reduction of nitrates to nitrites	-	+
ESCulin hydrolysis (β -glucosidase)	-	+
Assimilation of:		
L-arabinose	-	+
D-mannose	-	+
N-acetyl-glucosamine	-	+
potassium gluconate	-	+
API ZYM test for enzyme activity		
Alkaline phosphatase	+	-
Esterase	-	+
α -glucosidase	+	-
Major polar lipid profiles	DPG, PG	DPG, PG

Figures

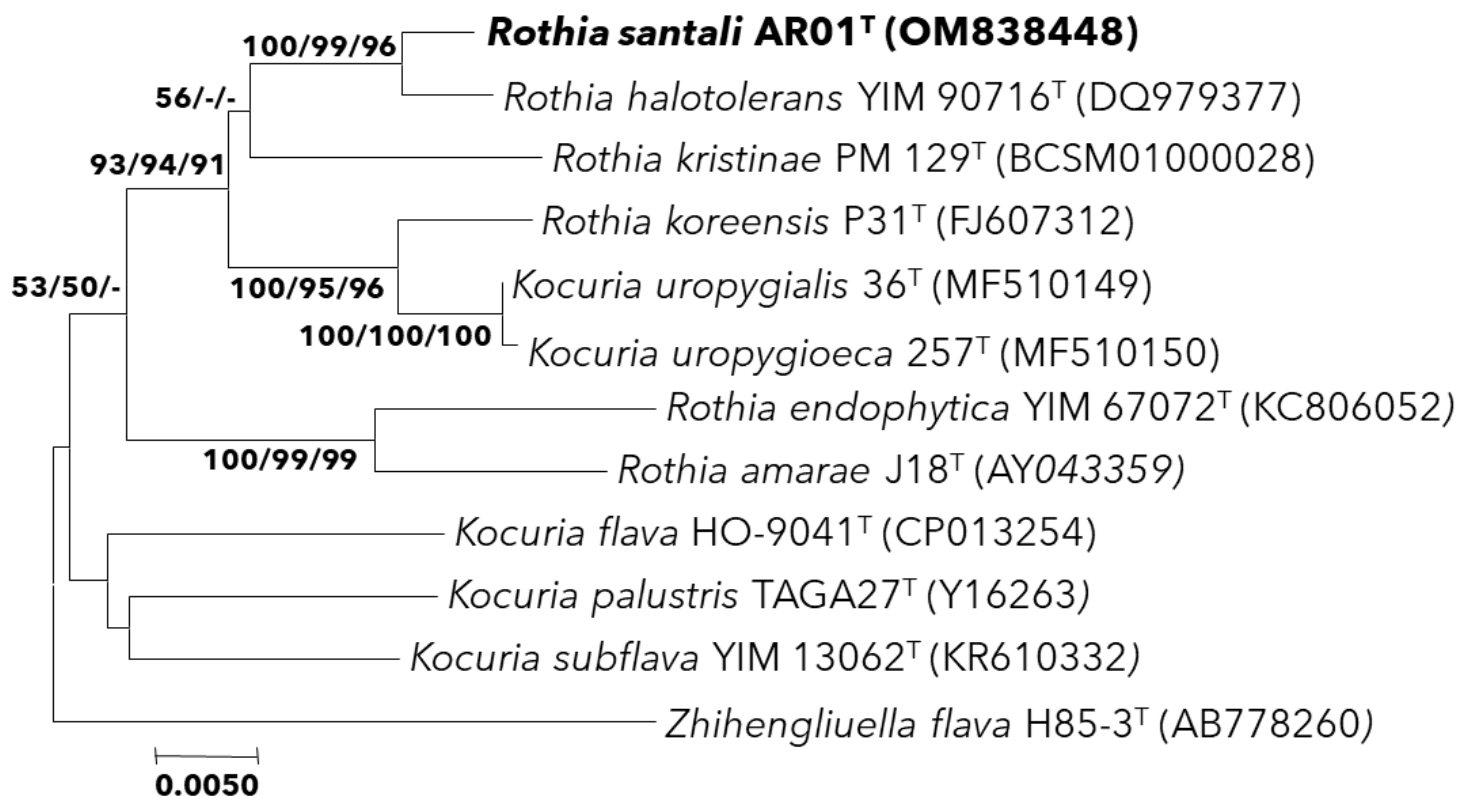


Figure 1

Phylogenetic tree constructed from the 16S rRNA gene sequences showing the relationship of strain AR01^T with other closely related members of the genus *Rothia* and *Kocuria* computed by neighbor-joining (NJ), maximum-likelihood (ML), and maximum parsimony (MP) methods for the phylogenetic tree-building using MEGA 7. The topologies of the trees were evaluated by bootstrap analysis based on 1000 replicates. Figures at nodes of the branches indicate the percentage of replicate trees obtained from the NJ, ML, and MP methods. The associated taxa clustered together in the bootstrap test. There were a total of 1386 positions in the final data set. The 16S rRNA gene sequence of *Zhihengliuella flava* H85-3 (AB778260) was used as an outgroup. Bar indicates the number of nucleotide substitutions per site.

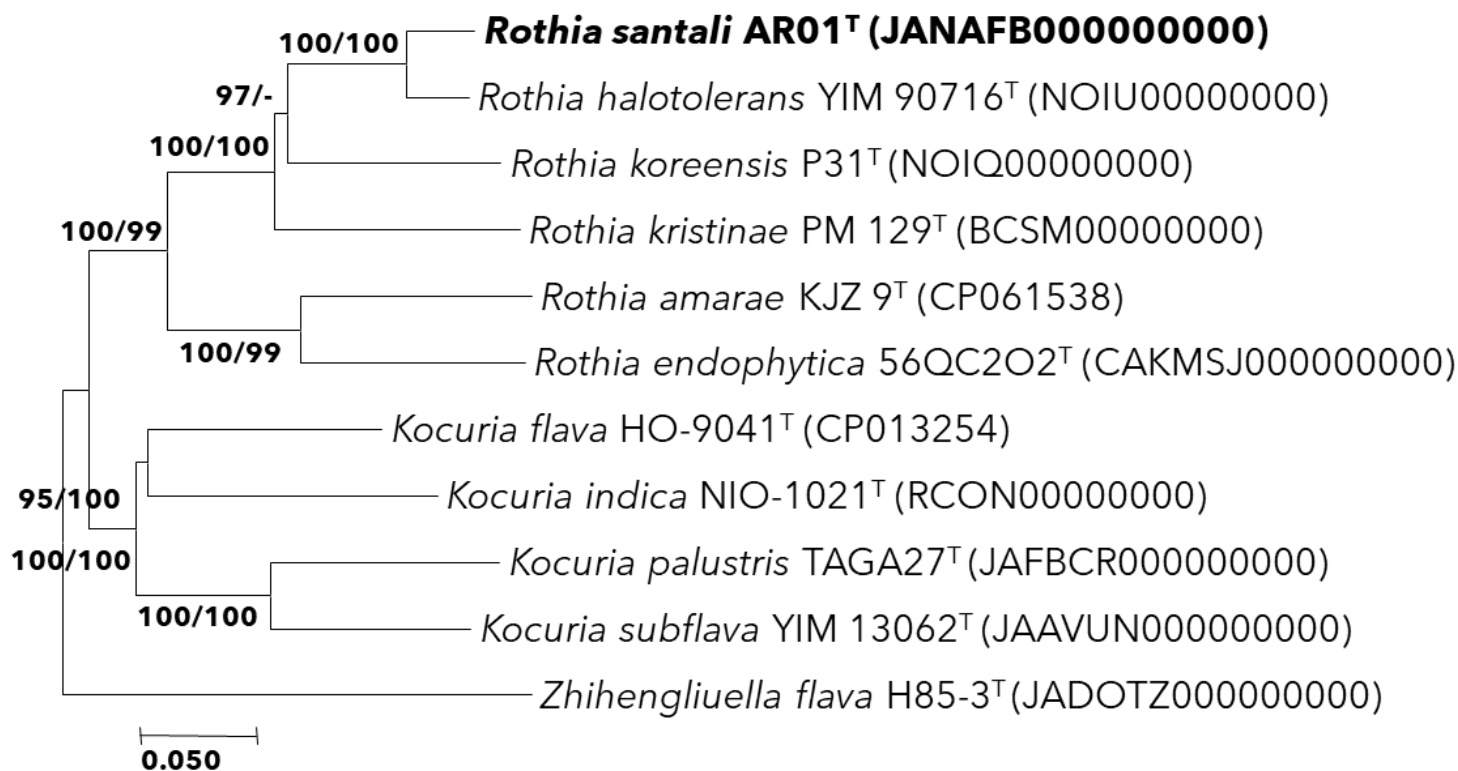


Figure 2

A combined pan-genome phylogenetic tree of strain AR01^T derived from the orthologous protein and gene sequences was constructed using BPGA and UBCG tools. The BPGA- and UBCG-based phylogenetic trees were reconstructed in MEGA 7 using the neighbor-joining method and maximum likelihood methods, respectively, at 1000 bootstrap replicates. Figures at branch points are bootstrap values observed in trees obtained using BPGA and UBCG, respectively. The draft genome sequence of *Zhihengliuella flava* H85-3^T (JADOTZ0000000000) was used as an out-group. Bar indicates the number of substitutions per site.

Figure 3

Venn diagram of protein orthologs (a) and the COG functional category distribution of genes (b) shared by *Rothia santali* AR01^T (MCC 4800), *Rothia halotolerans* YIM90716^T (KCTC 19172), *Rothia koreensis* P31^T (JCM 15915), and *Rothia kristinae* PM 129^T (NBRC 15354). The figures on the bars indicate a number of genes assigned to a particular functional category listed on the right side in the COG functional category distribution.

Supplementary Files

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