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Roseomonas hellenica sp. nov., isolated from roots of wild-growing *Alkanna tinctoria*

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ABSTRACT

Two Gram-negative, aerobic, rod-shaped and yellow-orange pigmented bacterial strains (LMG 31523^T and LMG 31524) were isolated from roots of wild-growing *Alkanna tinctoria* plants collected near Thessaloniki, Greece. Analysis of their 16S rRNA gene sequences revealed that they form a separate cluster related to the genus *Roseomonas*. A comparative whole genome analysis of the two strains and the type strains of related *Roseomonas* species revealed average nucleotide identity values from 78.84 and 80.32%. The G + C contents of the genomic DNA of strains LMG 31523^T and LMG 31524 were 69.69% and 69.74%, respectively. Combined data from phenotypic, phylogenetic and chemotaxonomic studies indicated that the strains LMG 31523^T and LMG 31524 represent a novel species of the genus *Roseomonas*. Genome analysis of the new strains showed a number of genes involved in survival in the rhizosphere environment and in plant colonization and confirmed the endophytic characteristics of LMG 31523^T and LMG 31524. Since the strains LMG 31523^T and LMG 31524 were isolated from a plant collected in Greece the name *Roseomonas hellenica* sp. nov. is proposed. The type strain is LMG 31523^T (=CECT 30032^T).

Introduction

The first *Roseomonas* strains were isolated by Gilardi and Faur [16] and described as relatives of *Methylobacterium*. The bacteria were isolated from clinical sources and were reported as pink-pigmented gram-negative coccobacilli bacteria, involved in human infections [16]. Later, Rihs et al. [40] proposed a new genus for these bacteria, establishing the genus *Roseomonas*. This genus is currently assigned to the family *Acetobacteraceae* in the class *Alphaproteobacteria* [45]. While the first species originated from clinical samples, more recently bacteria from the genus *Roseomonas* have been reported from various other environments, including air [49], soil [12,27,37,50], activated sludge [32], lake sediments [24], freshwater and drinking water [2,15], and plants [11,36]. *Roseomonas* species isolated from rhizosphere include *R. oryzicola* and *R. oryzae* from rice [8,38], *R. soli* from Chinese cabbage [27] and *R. hibiscisoli* from hibiscus [48]. Other plant-associated *Roseomonas* species were reported in banana (*R. musae*) and olive (*R. eleocarpi*) phyllospheres [36,11]. They were described as non-motile bacteria with respectively white and pink-pigmented colonies. At the time of the writing, 45 validly described species and two subspecies belong to the genus *Roseomonas* (http://www.bacterio.net/).

As part of a study of root endophytic bacteria from wild-growing *Alkanna tinctoria* (L.) Tausch (red alkanet) plants collected in Northern Greece, the strains LMG 31523^T and LMG 31524 were obtained as orangish bacterial colonies growing on the medium 1/10 869 supplemented with 0.32 mM allantoin after incubation at 20 °C for two weeks. Preliminary identification using partial 16S rRNA gene sequences indicated that LMG 31523^T and LMG 31524 have near identical 16S rRNA gene sequences related to the genus *Roseomonas*. In this study, the novel *Roseomonas*-like isolates were taxonomically characterized using a polyphasic approach and comparative genomics. Based on the results, we conclude that the two strains represent a novel species for which the name *Roseomonas hellenica* sp. nov. is proposed.

Methods

Isolation

Strains LMG 31523^T and LMG 31524 were isolated from roots of wild-growing *A. tinctoria* plants collected in December 2017, in

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Northern Greece, (Seich Sou area close to the Theatre of the Earth, Thessaloniki, Greece) under a special collection permit issued yearly by the Greek Ministry of Environment and Energy. Original plant material is maintained in the living *ex-situ* collections of the Institute of Plant Breeding and Genetic Resources, Agricultural Organization Demeter under the IPEN (International Plant Exchange Network) accession number GR-1–18,6081. To isolate the strains, pieces of roots were surface sterilized in a solution of 70% ethanol for 5 min followed by a rinse with sterile distilled water. The root was then sterilized with a solution of 1.4% of NaOCl for 20 min and rinsed again with sterile water. A last step was the immersion of the root in 2% $Na_2S_2O_3$ for 10 min to neutralize the effect of bleach, followed by a last rinse in sterile water. The root was then crushed in phosphate saline buffer. Dilutions to extinction were conducted and plated on 1/10 869 medium supplemented with 0.32 mM allantoin. After incubation at 20 °C for two weeks, orangish bacterial colonies were obtained and further purified. For routine cultivation, R2A medium and 28 °C as incubation temperature were used. Following primary isolation and purification, the strains were stored at −80 °C in R2B broth (Difco) supplemented with 15% (v/v) glycerol.

PCR-based 16S rRNA gene analysis

For determination of the near-complete 16S rRNA gene sequence, the genomic DNA was extracted by alkaline lysis according to the method of Niemann et al. [35]. Amplification of the 16S rRNA gene was performed using primers pA (5′-AGAGTTTGATCCTGGCTCAG- 3′, forward), hybridizing at positions 8–27 according to the *Escherichia coli* numbering system, and pH (5′ -AAGGAGGTGATCCAGCCGCA- 3′, reverse, positions 1541–1522) [9]. The PCR products were sequenced by Eurofins Genomics, Mix2seq service, with the primers BKL1 (5′-GTAT TACCGCGGCTGCTGGCA-3′, reverse, positions 536-516), *O (5′-AACTCAAAGGAATTGACGG-3′, forward, positions 928-936), gamma (5′-ACTGCTGCCTCCCGTAGGAG-3′, reverse, positions 358-339), *pD (5′-CAGCAGCCGCGGTAATAC-3′, forward, positions 519-536) [9], and the sequences obtained were identified with EzTaxon database [51].

Phylogenetic analyses

For analysis of the 16S rRNA gene, the complete 16S rRNA gene sequences of all *Roseomonas* type strains available on NCBI were downloaded from the NCBI database. The 16S rRNA gene sequence of *Acetobacter orleanensis* NBRC 13752^T was used as an outgroup. Sequences were aligned using MEGA7 [28]. Subsequently, phylogenetic neighbour-joining tree and maximum likelihood tree (1000 bootstraps) were reconstructed and visualized using the iTOL software [30].

MALDI-TOF MS profiling

The matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) was performed with the two strains as well as the related type strains *R. arctica* LMG 28251^T, *R. eburnea* LMG 31228^T, *R. oryzicola* LMG 31161^T, *R. alkalliterrae* LMG 31230^T, *R. soli* LMG 31231^T, *R. terrae* LMG 31159^T and *R. lacus* LMG 31229^T. The bacteria were grown on R2A medium and the cultures were subcultured twice before the analysis. Preparation of cell extracts and acquisition of bacterial fingerprints were performed according to Wieme et al. [47]. The resulting raw mass spectra were extracted as t2d files and converted into text files using the Flex Analysis (Bruker Daltonik GmbH). The text files were then imported in BioNumerics 7.5 (Applied Maths) and transformed into fingerprints. Finally, the similarity between fingerprints was determined using Pearson's product moment correlation after which spectra were clustered using unweighted pair group method with arithmetic mean (UPGMA).

Genome sequencing and analyses

To allow genome-based analyses, the genomes of strains LMG 31523^T and LMG 31524 were sequenced. Additionally, the type strains of related *Roseomonas* species for which no genomic sequence was available were sequenced (i.e., *R. arctica* LMG 28251^T, *R. eburnea* LMG 31228^T, *R. oryzicola* LMG 31161^T, *R. alkalliterrae* LMG 31230^T, *R. soli* LMG 31231^T, *R. terrae* LMG 31159^T). Genomic sequence for *R. lacus* (LMG 31229^T) was already publicly available. For sequencing, genomic DNA was extracted using an automated Maxwell DNA preparation instrument (Promega) following Tahon et al. [44]. Subsequently, the genomic sequences were determined using the Illumina HiSeq 2500 platform with 2 × 125 bp cycles at Baseclear. Trimmomatic was used for the trimming step [6]. The genomes were assembled using Shovill 0.9.0 (https://github.com/tseemann/shovill). The quast program was used to generate the summary statistics of the assembly (e.g., G + C content) [17]. For a genome-based phylogeny, the *Roseomonas* genomes were screened for the presence of 107 single-copy core genes, found in a majority of bacteria, using the automated bcgTree pipeline [1]. *Acetobacter orleanensis* NBRC 13752^T was used as an outgroup. The tree was then visualized using the iTOL software [30].

Comparative genome analysis was conducted using Orthofinder [13]. The genomic sequences were annotated with Eggnog [21], Kegg-Koala [25] and CAZy [33] databases.

The Bioprojects and Biosamples were deposited at DDBJ/ENA/GenBank (Table 1). Raw sequence data are available from the NCBI sequence read archive (www.ncbi. nlm. nih. gov/ sra) under the following accession numbers: SRR10982980 (LMG 28251^T), SRR10983219 (LMG 31159^T), SRR10983251 (LMG 31161^T), SRR10983440 (LMG 31230^T), SRR10983582 (LMG 31231^T), SRR10985188 (LMG 31228^T), SRR11004557 (LMG 31523^T) and SRR11004675 (LMG 31524).

Morphology and metabolic profile

After incubation on R2A at 28 °C for 72 h, cell morphology was observed using a stereomicroscope. Gram staining was performed as described by MacFaddin [34]. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined using 1 % (w/v) tetramethyl p-phenylenediamine. Motility was evaluated using the hanging drop technique [5]. Metabolic profiling of the strains was performed at 28 °C in triplicate. The growth of the strains was tested at 0, 4, 10, 15, 20, 25, 30, 35, 40 and 45 °C on R2A medium for seven days. Salt tolerance was evaluated in R2B supplemented with 0 to 10% (w/v) NaCl with 1% interval. The pH range for growth was tested at pH 4–11 with 1 unit interval in R2B. To determine pH and salt tolerance ranges and optima, growth was monitored by measuring the optical density at 600 nm every day during five days. Growth in anaerobic atmosphere was also determined on R2A in Anaerocult A system (Merck) for 10 days at 28 °C.

The ability of the strains to hydrolyse casein was determined on R2A supplemented with 13 g/L of skim milk. Hydrolysis of Tween 20 and 80 was tested on a medium composed of 10 g/L peptone, 0.1 g/L $CaCl_2$, 2 H_2O, 9 g/L agar, pH 7.4 with the addition of sterile Tween after autoclaving at the final concentration of 1%. The hydrolysis of carboxymethyl-cellulose (CMC) was tested by adding a solution of 0.2% Congo red on R2A supplemented with 0.1% CMC. Hydrolysis of starch was tested on R2A supplemented with 10 g/L starch with addition of Lugol's iodine solution to reveal a clear halo

Table 1
General characteristics of genomes from R. hellenica LMG 31523^T, LMG 31524, R. arctica LMG 28251^T, R. terrae LMG 31159^T, R. oryzicola LMG 31161^T R. eburnea LMG 31228^T, R. lacus LMG 31229^T, R. alkalliterrae LMG 31230^T, R. soli LMG 31231^T.

Characteristics	Bioproject	Biosample	Predicted genes (unique)	G + C (%)	N50	Total length (>= 0 bp)	Contigs (>= 0 bp)
LMG 31523^T	PRJNA603457	SAMN13932463	6845	69.69	127kbp	7.3Mbp	161
LMG 31524	PRJNA603459	SAMN13932470	6870	69.74	79kbp	7.2Mbp	202
LMG 28251^T	PRJNA603473	SAMN13932596	4194	69.49	158kbp	4.4Mbp	51
LMG 31159^T	PRJNA603472	SAMN13932595	5477	69.25	180kbp	5.8Mbp	67
LMG 31161^T	PRJNA603469	SAMN13932592	5165	71.23	69kbp	5.3Mbp	161
LMG 31228^T	PRJNA603468	SAMN13932575	5489	71.21	162kbp	5.9Mbp	89
LMG 31229^T	PRJDB10509	SAMD00245122	6032	68.73	215kbp	6.4Mbp	93
LMG 31230^T	PRJNA603471	SAMN13932594	4432	72.58	16kbp	4.3Mbp	526
LMG 31231^T	PRJNA603470	SAMN13932593	5068	70.83	48kbp	5.2Mbp	216

of hydrolysis. The ability of the strains to hydrolyse tyrosine was determined on medium consisting of 5 g/L peptone, 3 g/L meat extract, 20 g/L agar, 5 g/L tyrosine. A bright halo around the bacterial colonies indicated positive activity. For the hydrolysis of gelatine, medium with 3 g/L meat extract, 5 g/L peptone, 120 g/L gelatine was used. After incubation, the plates were stored in the fridge for 30 min. Strains for which the medium was not solid anymore were considered positive for gelatinase activity [10].

Antibiotic sensitivity was studied by growing the strains on R2A and using antimicrobial susceptibility paper discs [3] saturated with the following antibiotics (Oxoid): chloramphenicol (30 μg/disc), tetracycline (30 μg/disc), vancomycin (30 μg/disc), amikacin (30 μg/disc), streptomycin (10 μg/disc), ampicillin (10 μg/disc), novobiocin (5 μg/disc).

Other physiological and biochemical activities were investigated using API 50CH, API ZYM, API 20E and API 20NE kits (bioMerieux, France) as recommended by the manufacturer. After inoculation, the galleries were incubated at 28 °C and reactions were checked every day. However, because of the relatively slow growth of some of the reference strains, for the API 50CH, API 20E and API 20NE assays the time of incubation was extended to 14 days with galleries wrapped in a plastic bag to limit dehydration.

Availability of biological material

The type strain, originally labelled R-73080, was deposited in the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG, Ghent, Belgium), and the Spanish Type Culture Collection (CECT) under the accession numbers LMG 31523^T and CECT 30032^T, respectively. Additionally, strain R-73070 was deposited in the BCCM/LMG collection as LMG 31524.

Results and discussion

Phylogenetic placement and phylogenomics

The phylogenetic comparison of the nearly complete 16S rRNA gene sequences of LMG 31523^T and LMG 31524 with data from EzTaxon revealed that the strains are related to the genus *Roseomonas* with a similarity of 99.92% to each other. The highest similarity was obtained with the type strain of *R. arctica* (sequence similarity 97.17%). Additionally, phylogenetic neighbour-joining and maximum likehood analyses showed similar results forming a robust cluster within the genus *Roseomonas*. The results presented in the maximum likehood tree (Fig. 1) indicate that the two new strains could represent a new species in the genus *Roseomonas*. The most closely related type strains are *R. arctica* LMG 28251^T, *R. eburnea* LMG 31228^T, *R. lacus* LMG 31229^T, *R. terrae* LMG 31159^T, *R. alkaliterrae* LMG 31230^T, *R. soli* LMG 31231^T, *R. oryzicola* LMG 31161^T and *R. sediminicola* FW-3^T.

Clustering of the MALDI-TOF MS fingerprints showed that the two strains LMG 31523^T and LMG 31524 formed a separate cluster distinct from the profiles of the type strains of related *Roseomonas* species selected for this analysis (Fig. S1, panel A). This confirms the results of the phylogenetic comparison of the nearly complete 16S rRNA gene and indicates that LMG 31523^T and LMG 31524 represent a novel species in the genus *Roseomonas*.

Phylogenomic analysis was conducted on the sequences of strains LMG 31523^T, LMG 31524 and related type strains with the exception of *R. sediminicola* FW-3^T [19] that was not validly published at the time of the analysis. The genome sequences of strains LMG 31523^T, LMG 31524, LMG 28251^T, LMG 31159^T, LMG 31161^T, LMG 31228^T, LMG 31230^T, LMG 31231^T were deposited in GenBank under the accession number JAAGBB000000000, JAAGBC000000000, JAAEDH000000000, JAAEDI000000000, JAAEDK000000000, JAAEDL000000000, JAAEDJ000000000 and JAAEDM000000000, respectively. Analysis of a set of 107 single-copy core genes, found in the majority of bacteria [1], confirmed the placement of the new strains in the genus *Roseomonas* (Fig. 2). Genomic data also allowed the calculation of ANI. For species delineation, the generally accepted cut-off for ANI is 95%. The ANI values for the strains LMG 31523^T, LMG 31524 and the type strains were between 78.84 and 80.32% (Table 2). These data are below the recommended threshold, thus indicating that the isolates constitute a novel species of the genus *Roseomonas*. Strains LMG 31523^T and LMG 31524 shared an ANI value of 97.80%, showing that they are two different strains from the same new species in the genus *Roseomonas* [23]. Moreover, the study of the MALDI-TOF MS fingerprints of the two strains LMG 31523^T and LMG 31524 indeed showed differences between the two spectra (Fig S1, panel B), confirming that they are two different strains from the same new species in the genus *Roseomonas*.

Morphology and metabolic profile

The colonies of LMG 31523^T and LMG 31524 on R2A medium are orangish, circular, raised and smooth. Although some other *Roseomonas* species with orange colonies have been described previously, the colony colour of the closest related type strains is different: *R. arctica* LMG 28251^T, *R. oryzicola* LMG 31161^T, *R. alkalliterrae* LMG 31230^T and *R. terrae* LMG 31159^T produce pinkish colonies, whilst *R. eburnea* LMG 31228^T, *R. soli* LMG 31231^T and *R. lacus* LMG 31229^T produce whitish colonies. The strains are Gram-negative, motile, aerobic bacteria. Other phenotypic characteristics are presented in Table 3.

In the API 20NE system, LMG 31524 and LMG 31523^T hydrolysed urea and use arabinose, adipate and malate. They were able to assimilate potassium gluconate. Reference strains *R. eburnea* LMG 31228^T and *R. oryzicola* LMG 31161^T used adipate and malate. *R. soli* LMG 31231^T metabolized arabinose, malate and capric acid whereas *R. lacus* LMG 31229^T used only malate.

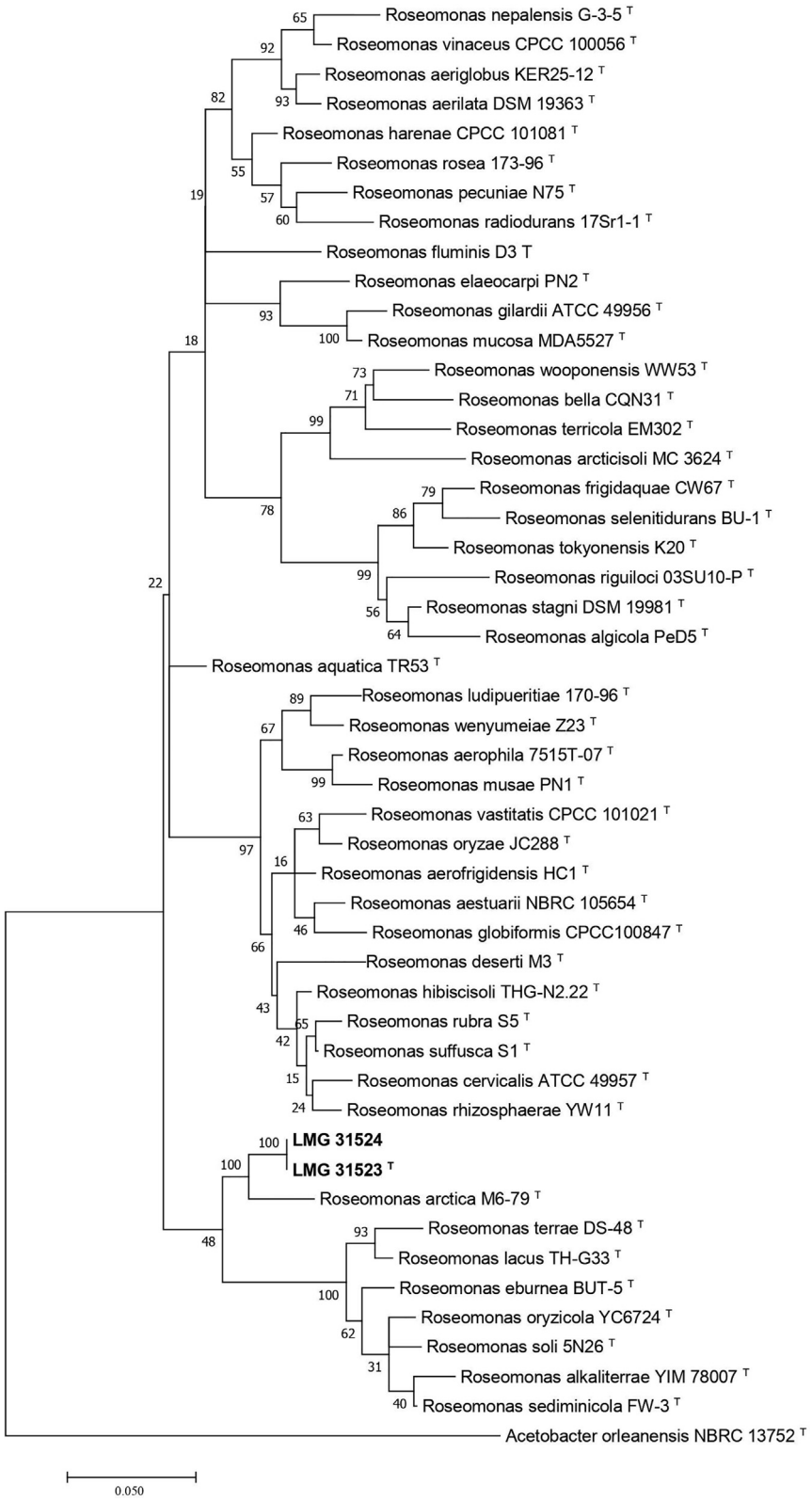

Fig. 1. Maximum-likehood phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strains LMG 31524 and LMG 31523^T and related taxa. Numbers at nodes are percentage bootstrap values based on 1000 replications. T means type strains. Acetobacter orleanensis NBRC 13752^T was used as an outgroup.

In the API 20E system, assimilation of L-leucine, citrate, urea hydrolysis was positive and tryptophane deaminase activity was weak for LMG 31524 and LMG 31523^T. For the latter strain also a weak fermentation activity for glucose, L-rhamnose, D-melibiose and L-arabinose was observed. *R. terrae* LMG 31159^T and *R. lacus* LMG 31229^T hydrolysed urea.

In the API ZYM system, LMG 31524 expressed esterase, esterase lipase, leucine arylamidase, naphtol and acid phosphatase activity,

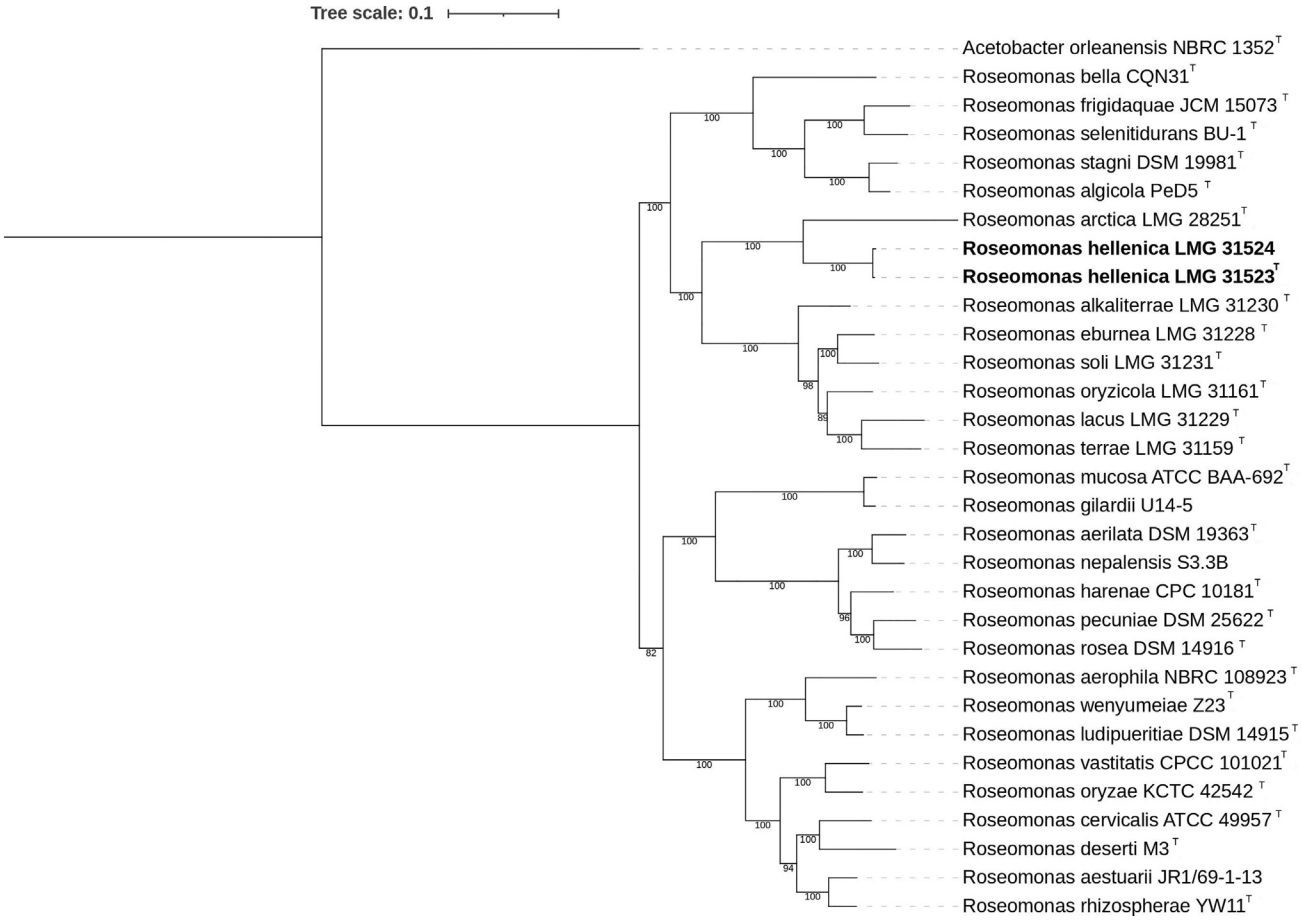

Fig. 2. Bcg tree based on assembled gene sequences, showing the phylogenetic relationships of strains LMG 31524 and LMG 31523^T and related taxa. Numbers at nodes are percentage bootstrap values based on 1000 replications. T means type strains. Acetobacter orleanensis NBRC 13752^T was used as an outgroup.

Table 2
Results (%) of average nucleotide identity (ANI) between genomes of species from Roseomonas genus. LMG 31523^T, LMG 31524 and type strains of closely related species of the genus Roseomonas: R. arctica LMG 28251^T, R. terrae LMG 31159^T, R. oryzicola LMG 31161^T R. eburnea LMG 31228^T, R. lacus LMG 31229^T, R. alkalliterrae LMG 31230^T, R. soli LMG 31231^T.

Characteristics	LMG 31523^T	LMG 31524	LMG 28251^T	LMG 31159^T	LMG 31161^T	LMG 31228^T	LMG 31229^T	LMG 31230^T	LMG 31231^T
LMG 31523^T		97.80	80.17	78.70	79.65	79.96	78.90	79.74	79.40
LMG 31524			80.32	78.75	79.73	79.95	78.84	79.77	79.57
LMG 28251^T				78.05	79.11	79.05	78.41	79.25	78.62
LMG 31159^T					82.93	82.94	82.92	82.56	82.65
LMG 31161^T						85.09	83.25	84.85	85.14
LMG 31228^T							83.22	84.97	86.83
LMG 31229^T								82.54	82.83
LMG 31230^T									84.73
LMG 31231^T									

whereas LMG 31523^T produced esterase lipase, lipase, leucine aminopeptidase, chymotrypsin, cysteine aminopeptidase, phosphohydrolase, α-galactosidase. Reference strains *R. arctica* LMG 28251^T, *R. lacus* LMG 31229^T, *R. soli* LMG 31231^T, *R. terrae* LMG 31159^T and *R. oryzicola* LMG 31161^T expressed alkaline phosphatase, acid phosphatase esterase and naphtol degradation activity. *R. alkaliterrae* LMG 31230^T expressed the same activities but did not produce alkaline phosphatase. *R. arctica* LMG 28251^T, *R. alkaliterrae* LMG 31230^T, *R. soli* LMG 31231^T, *R. oryzicola* LMG 31161^T and *R. eburnea* LMG 31228^T produced esterase lipase. *R. arctica* LMG 28251^T and *R. eburnea* LMG 31228^T showed also lipase activity. *R. terrae* LMG 31159^T and *R. oryzicola* LMG 31161^T expressed leucine aminopeptidase activity, whereas *R. eburnea* LMG 31228^T expressed cysteine aminopeptidase, phosphohydrolase, α-galactosidase, and chymotrypsin activities.

In API 50CH, all tested strains hydrolysed esculin, LMG 31523^T most strongly. The strains LMG 31523^T and LMG 31524 were able to ferment D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-glucose, D-mannose, L-rhamnose and D-fucose. LMG 31523^T also metabolizes erythritol, D-adonitol, amygdalin, salicin, and D-trehalose. The results of the reference strains can be found Table 3.

Strains LMG 31524 and LMG 31523^T were sensitive to tetracyclin and amykacin but resistant to vancomycin, streptomycin, ampicillin and novobiocin. Strain LMG 31524 was sensitive to chloramphenicol whereas LMG 31523^T was resistant (Table 3).

We observed some differences in the metabolic profiles compared to previously published results for some of the reference strains. *R. arctica* LMG 28251^T was resistant to vancomycin and novobiocin whereas it was originally described as sensitive [37]. Such contrast can be linked with the cultivation medium. Indeed,

Table 3
Phenotypic characteristics of strains R. hellenica LMG 31523^T, LMG 31524 and type strains of related species of the genus Roseomonas: R. arctica LMG 28251^T, R. terrae LMG 31159^T, R. oryzicola LMG 31161^T R.eburnea LMG 31228^T, R. lacus LMG 31229^T, R. alkalliterrae LMG 31230^T, R. soli LMG 31231^T, R. sediminicola FW-3^T. *data from [19]. +, positive; −, negative; R, resistant; S, sensitive.

Characteristics	LMG 31523^T	LMG 31524	LMG 28251^T	LMG 31159^T	LMG 31161^T	LMG 31228^T	LMG 31229^T	LMG 31230^T	LMG 31231^T	FW-3^{T*}
Anaerobic growth	−	−	−	−	−	−	−	−	−	−
Oxidase	−	−	−	−	−	−	−	−	−	+
Catalase	+	+	+	+	+	+	+	+	+	+
Growth conditions:										
Temperature range (°C)	4–45	4–45	4–45	4–45	4–45	4–45	4–45	4–45	4–40	10–37
Temperature optimum (°C)	25–30	25–30	15–25	25	30–35	25–30	25–30	25–30	25–30	25–37
Salinity range (% NaCl, w/v)	0–10	0–10	0	0–6	0–10	0–10	0–10	0–10	0–10	0–1
Salinity optimum (% NaCl, w/v)	0–2	0–2	0	0–3	0–3	0–2	0–3	0–3	0–3	0–1
pH range	4–11	4–11	4–10	4–11	4–11	4–11	4–11	4–11	4–11	5.5–10
pH optimum	7–8	7–8	6–8	7–8	7–8	6–8	6–8	7	7–8	7
Hydrolysis of:										
Casein	−	−	−	−	−	+	−	−	−	−
Tween 20	−	−	−	−	−	−	−	−	−	
Tween 80	+	+	−	−	−	−	−	−	−	−
CMC/Gelatin	−	−	−	−	−	−	−	−	−	
Starch	−	−	−	−	−	−	−	−	−	−
Tyrosine	+	+	+	−	−	−	−	−	−	−
Utilisation of:										
Erythritol	+	−	−	−	−	−	−	−	−	
D-arabinose/L-arabinose/D-ribose	+	+	+	−	−	−	−	−	−	
D-xylose/L-xylose	+	+	−	−	−	−	−	−	−	
D-adonitol	+	−	−	−	−	−	−	−	−	
D-glucose	+	+	−	−	−	−	−	−	−	
D-mannose/L-rhamnose	+	+	−	−	−	−	−	+	+	
L-sorbose	−	−	−	−	−	−	−	+	+	
Amygdalin	+	−	−	−	−	−	−	−	−	
Arbutin	−	−	+	−	−	−	−	−	−	
Esculin ferric citrate	+	+	+	+	+	+	+	+	+	
Salicin	+	−	+	−	−	−	−	−	−	
D-trehalose	+	−	−	−	−	−	−	−	−	
Starch	−	−	−	−	−	−	−	+	+	
D-lyxose	−	−	+	−	−	−	−	+	+	
D-tagatose	−	−	−	−	−	−	−	+	+	
D-fucose	+	+	−	−	−	−	−	+	+	
L-fucose	−	−	−	−	−	−	−	−	+	
Potassium 5-ketogluconate	−	−	−	−	+	−	−	+	+	
Susceptibility to:										
Chloramphenicol (30 μg/disc)	R	S	S	S	S	S	S	S	R	S
Vancomycin (30 μg/disc)	R	R	R	R	R	S	R	R	R	R
Streptomycin (30 μg/disc)	R	R	S	S	R	S	S	S	S	
Novobiocin (30 μg/disc)	R	S	R	S	S	S	R	S	S	
Ampicillin (30 μg/disc)	R	R	S	S	S	S	R	S	S	
Tetracycline (30 μg/disc)	S	S	S	S	S	S	S	S	R	
Amykacin (30 μg/disc)	S	S	R	S	R	S	S	R	S	

R. arctica LMG 28251^T was grown on R2A medium in this study and not on Nutrient agar. We also observed salt tolerance up to 10% NaCl (w/v) for most of the strains (with an optimum growth at 2–3%) whereas most of the reference strains were described to resist only to 1% NaCl. The evaluations of salt resistance as well as the API tests are qualitative, and thus can be subject to differences in interpretation. Moreover, the initial concentration of inoculum as well as differences in the time of incubation might contribute to different results.

General genome characteristics of Roseomonas species

The genomes of the following strains of the genus *Roseomonas* were analysed for general genome characteristics: *R. hellenica* strains LMG 31523^T and LMG 31524, *R. arctica* LMG 28251^T, *R. eburnea* LMG 31228^T, *R. oryzicola* LMG 31161^T, *R. alkalliterrae* LMG 31230^T, *R. soli* LMG 31231^T, *R. terrae* LMG 31159^T, *R. lacus* LMG 31229^T. The characteristics of the *Roseomonas* genomes studied are listed in Table 1. Coverage was 100 × for all genomes. The final assemblies and their quality are in agreement with the minimal standards for the use of genome data as proposed by Chun et al. [7]. The use of bcgTree showed that LMG 31230^T, LMG 31231^T, and LMG 31524 contained 106 single-copy core genes on 107 whereas the other reference strains and LMG 31523^T contained all the 107 single-copy core genes included in the bcgTree data set [1]. Therefore, it can be assumed that the genome sequences are near-complete. Genome sizes were not in the same range with much larger genomes for LMG 31523^T and LMG 31524 (7.2–7.3 Mbp) compared to the related reference strains (4.4 to 6.4 Mbp; Table 1). Genomes of 50 *Roseomonas* strains available in the NCBI database have genomes size ranging from 4.13 to 6.78 Mb. The observation that our new isolates have a larger genome size can be related to endophyte-specific functions (see below) and is in agreement with Levy et al. (2017) [31] who compared genomic features of a large number of plant-associated bacteria. The DNA G + C content of LMG 31523^T and LMG 31524 were 69.69% and 69.74% respectively, which is within the range known for the genus *Roseomonas* (65.8–73.0 mol%) [26,41].

G + C content as determined here by using whole genome sequences generally showed small differences with the G + C content as previously determined by the thermal denaturation method in the *Roseomonas* species characterization [8,12,24,27,32,37,50].

However, the G + C content using whole genome sequences is higher for *R. eburnea* LMG 31228^T [32] and *R. soli* LMG 31231^T [27] with a difference of 3.61 mol% and of 2.53 mol% respectively.

Comparative genome analysis of Roseomonas species

This study is the first comparative genomic analysis of *Roseomonas* species. The genomes of the following strains of the genus *Roseomonas* allow a first insight in the (core) metabolism of these organisms: strains LMG 31523^T and LMG 31524, *R. arctica* LMG 28251^T, *R. eburnea* LMG 31228^T, *R. oryzicola* LMG 31161^T, *R. alkalliterrae* LMG 31230^T, *R. soli* LMG 31231^T, *R. terrae* LMG 31159^T, *R. lacus* LMG 31229^T. Using OrthoFinder, the core genome of *Roseomonas* was found to consist of 2151 proteins. Based on the annotated genome sequences, the central metabolism of *Roseomonas* species very likely involves the glycolysis, tricarboxylic acid cycle and the pentose phosphate pathway (Supp. Table S1-S3). The absence of some enzymes of the pentose phosphate pathway in the annotated genomes suggests that this pathway cannot supply certain building blocks and NADPH which may be used for other biosynthetic processes. All the key enzymes required to convert the products of the tryptophan metabolism into NADPH are present in the nicotinate and nicotinamide pathway and may indicate that this pathway is preferably used to produce NADPH (Supp. Table S4). Key enzymes for carbon fixation are present in all genomes except in *R. alkalliterrae* LMG 31230^T.

Roseomonas is a genus present in various environments such as air [49], plants [11,36], water [2,15], soil [12,27,37,50], human blood and the built environment [22,40]. This observation suggests the ability to use a wide diversity of compounds. The analysis of the genome for sulphur metabolism revealed that all type strains possess genes required for the conversion of taurine, methanosulfonate and thiosulfate into sulfite and then to the amino acid cysteine (Supp. Table S5). Thus, several substrates may be used by the bacteria to cover their need in sulphur. The presence of a nitrate/nitrite transporter in the genomes of all type strains suggests that these organisms can potentially also rely on the uptake of extracellular nitrate and nitrite. Additionally, the presence of NarGHI, NasA and NirB/D and NirA in the annotated genomes, except for *R. alkalliterrae* LMG 31230^T, indicates that these organisms may also be capable of converting nitrate to nitrite and nitrite to ammonia, respectively (Supp. Table S11). All type strains, except LMG 31230^T, contained genes involved in the conversion of nitrate to ammonia and then to glutamate and glutamine in their genomes. Moreover, a key enzyme, a carbon–nitrogen hydrolase (3.5.5.1), associated with the conversion of nitrile to ammonia is also present in all type strains (Supp. Table S11). Nitriles are present in several plant pathways such as the biosynthesis of cyanolipids and cyanogenic glycosides or the breakdown of glucosinolates [20]. Moreover, genomes of all type strains have genes implied in the metabolism of organic phosphates but only LMG *R. arctica* 28251^T, *R. oryzicola* LMG 31161^T and the new species represented by strains LMG 31523^T and LMG 31524 possess genes coding for inorganic phosphate transporter (Supp. Table S6). This attribute might confer a competitive advantage to LMG 31523^T and LMG 31524 for nutrient acquisition in the rhizosphere environment.

Given the endophytic origin of the strains, their ability to survive in the rhizosphere and to colonize plants may give them a competitive advantage in the environment. Therefore, the genomes were screened for the presence of genes involved in these processes. Using the KEGG-koala database [25], we found that only the genomes of LMG 31523^T and LMG 31524 contained an enzyme able to convert sucrose into fructose and glucose (Supp. Table S10). Sucrose is the most common form of carbohydrate used to transport carbon within a plant and is thus a great source of carbon for rhizospheric bacteria able to use it [29,39]. The genomes of LMG 31523^T and LMG 31524 are also enriched in siderophore receptors compared to the reference strains (Supp. Table S8). Siderophores are low molecular weight organic chelators with a very high and specific affinity for Fe(III). To support their growth, the

Table 4
Description of Roseomonas hellenica type strain LMG 31523^T.

Genus name	*Roseomonas*
Species name	*Roseomonas hellenica*
Specific epithet	*hellenica*
Species status	sp. nov
Species etymology	hel-lé-ni-ka. Gr. adj. ellenikos, Greek, N.L. fem. adj. hellenica, Greek, pertaining to Greece from where the bacterium was first isolated).
Description of the new taxon and diagnostic traits	Aerobic, motile bacterium. Colonies on R2A medium are orangish, circular, raised and smooth. Gram-negative staining, aerobic and motile. The temperature range for growth is 10 °C-40 °C, with an optimum at 20–35 °C. The pH range for growth is 4–11 with an optimum at pH 6–8. Growth in the absence of NaCl (range 0–2% NaCl).Able to hydrolyse esculin and urea and to use arabinose, adipate, malate and potassium gluconate. Assimilation of L-leucine, citrate and weak tryptophan deaminase activity. Able to ferment D-glucose, L-rhamnose, D-melibiose, L-arabinose. D-arabinose, D-ribose, D-xylose, L-xylose, D-glucose and D-mannose.The strains are sensitive to chloramphenicol, tetracycline and amykacin but resistant to vancomycin, streptomycin, ampicillin and novobiocin.
Country of origin	Greece
Region of origin	Thessaloniki
Date of isolation (dd/mm/yyyy)	03/2018
Source of isolation	Roots from *Alkanna tinctoria*
Sampling date (dd/mm/yyyy)	12/2017
Latitude (xx°xx′xx″N/S)	40° 37′ 53.0″N
Longitude (xx°xx′xx″E/W)	22° 58′ 18.1″E
16S rRNA gene accession nr.	MN647549
Genome accession number [RefSeq; EMBL; . . .]	JAAGBB000000000
Genome status	incomplete
Genome size	127 kbp
GC mol%	69.69%
Number of strains in study	2
Source of isolation of non-type strains	Roots from *Alkanna tinctoria*
Information related to the Nagoya Protocol	Permit no. 154553/1861/13-7-2017, Greece, issued by Greek Ministry of Environment and Energy
Designation of the Type Strain	R-73080
Strain Collection Numbers	LMG 31523^T and CECT 30032^T

production of siderophores confers an indisputable advantage to soil bacteria [4,14]. The presence of such genes in LMG 31523^T and LMG 31524 might help them to support their growth and provide a competitive advantage to survive, among other microbes, in the rhizosphere.

The plant colonization by bacteria depends on the interactions between the two organisms and is strongly dependent on chemotaxis processes. A bacterium with chemotaxis abilities is more likely to be a competent endophyte [18]. Strains LMG 31523^T and LMG 31524 encode in their genome all the key proteins for chemotaxis, including CheC and MCP, contrary to the reference strains that miss key genes (Supp. Table S7). The strains LMG 31523^T and LMG 31524 are also highly enriched in genes related with the bacterial secretion system type III (T3SS) (Supp. Table S9). The type III secretion system is usually associated with pathogenicity even though it is found in many Gram-negative bacteria, including non-pathogenic. Indeed, a defective T3SS in bacterial pathogens often results in non-pathogenic behaviour. The T3SS acts as a needle to infect eukaryotic cells and transfer proteins to the cells that help the bacteria to survive in the host and to escape immune responses [42,43,46,52]. The presence of several genes linked with the T3SS might be associated with a higher ability to colonize plants, whatever the potential pathogenic characteristic of the bacteria, and thus may be related with endophytic behaviour.

Moreover, compared to the genomes of the reference strains, the genomes of LMG 31523^T and LMG 31524 contain two extra genes involved in hemicellulose metabolism and one extra gene involved in the degradation of cellulose to cellobiose. They were also the only strains possessing potential pectinase activities (Supp. Table S10). Cellulose, pectin, and hemicellulose are constituents of the plant tissues. Enzymes involved in metabolism of such molecules are essential for plant colonization [18].

The genome analysis of strains LMG 31523^T and LMG 31524 thus seems to support the endophytic nature of this novel species of the genus *Roseomonas.*

The phenotypic characteristics and phylogenetic analyses described above indicate that strains LMG 31523^T and LMG 31524 represent a new species of the genus *Roseomonas*, for which the name *Roseomonas hellenica* sp. nov. is proposed (Table 4).

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CRediT authorship contribution statement

Angélique Rat: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Henry D. Naranjo:** Software, Validation. **Liesbeth Lebbe:** Investigation, Validation. **Margo Cnockaert:** Investigation, Validation. **Nikos Krigas:** Resources, Writing - review & editing. **Katerina Grigoriadou:** Resources, Writing - review & editing. **Eleni Maloupa:** Resources, Writing - review & editing. **Anne Willems:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2021.126206.

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