**Belnapia mucosa** sp. nov. and **Belnapia arida** sp. nov., isolated from desert biocrust

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**Abstract**

Two novel Gram-staining-negative, aerobic, cocci-shaped, non-motile, non-spore forming, pink-pigmented bacteria designated strains T6T and T18T, were isolated from a biocrust (biological soil crust) sample from the vicinity of the Tabernas Desert (Spain). Both strains were catalase-positive and oxidase-negative, and grew under mesophilic, neutrophilic and non-halophilic conditions. According to the 16S rRNA gene sequences, strains T6T and T18T showed similarities with Belnapia rosea CMGCC 1.10758T and Belnapia moabensis CP2CT (98.11 and 98.55% gene sequence similarity, respectively). The DNA G+C content was 69.80 and 68.96% for strains T6T and T18T, respectively; the average nucleotide identity by blast (ANIb) and digital DNA–DNA hybridization (dDDH) values confirmed their adscription to two novel species within the genus Belnapia. The predominant fatty acids were summed feature 8 (C\textsubscript{16:1}ω7c/C\textsubscript{16:1}ω6c), C\textsubscript{16:0}, C\textsubscript{18:1} 2-OH and summed feature 3 (C\textsubscript{16:1}ω7c/C\textsubscript{16:1}ω6c). According to the results of the polyphasic study, strains T6T and T18T represent two novel species in the genus Belnapia (which currently includes only three species), for which names Belnapia mucosa sp. nov. (type strain T6T = CECT 30228T=DSM 112073T) and Belnapia arida sp. nov. (type strain T18T=CECT 30229T=DSM 112074T) are proposed, respectively.

The genus Belnapia was first described by Reddy et al. [1] and it is, at the time of writing, comprised of three species, which were all isolated from soil samples: Belnapia moabensis [1], Belnapia rosea [2] and Belnapia soli [3]. In this study we describe the polyphasic characterization of two strains, namely T6T and T18T, which were isolated from biocrust (biological soil crust) samples from south-eastern Spain during a study on the microbial diversity of European arid regions.

Strains T6T and T18T were isolated in the vicinity of the Tabernas Desert (Almería, Spain) during a study on the culturable microbial diversity in European drylands [4]. The Tabernas Desert is considered the only arid desert in Europe [5]. In this study, the strains were isolated from desert biocrust samples obtained from near the Tabernas Desert Parc (37.002404° N, 2.450655° W) and homogenized in phosphate buffered saline (PBS; NaCl 8.0 g l−1, KCl 0.2 g l−1, Na\textsubscript{2}HPO\textsubscript{4} 1.44 g l−1, KH\textsubscript{2}PO\textsubscript{4} 0.24 g l−1) pH 7.4 (1 g in 1 ml). The suspensions were then spread on 1, 0.1 and 0.01× TSA plates. The plates were incubated at 23 °C for 1 week. Strain T6T was isolated from 0.1× TSA plates, whereas T18T was isolated from a 0.01× TSA plate. The isolation of the strains was carried out by re-streaking on fresh media until a pure culture was obtained. Cell suspensions in TSA and R2A were cryopreserved at −80 °C with 15% glycerol (v/v). Their taxonomic status was determined by a polyphasic approach. On the basis of the results from phylogenetic, phenotypic and chemotaxonomic analysis, it is concluded that strains T6T and T18T are related to members of the genus Belnapia and representatives of two novel species. In the present work, the predominant fatty acids were

trypticase soy agar (TSA; 15 g l\textsuperscript{-1} tryptone, 5 g l\textsuperscript{-1} NaCl, 5 g l\textsuperscript{-1} soya peptone), and Reasoner’s 2A Agar (R2A; 1 g l\textsuperscript{-1} peptone, 0.5 g l\textsuperscript{-1} yeast extract, 0.5 g l\textsuperscript{-1} dextrose, 0.3 g l\textsuperscript{-1} dipotassium phosphate, 0.05 g l\textsuperscript{-1} magnesium sulphate heptahydrate, 0.3 g l\textsuperscript{-1} sodium pyruvate). Agar was autoclaved separately and added before plating at a final concentration of 15 g l\textsuperscript{-1}. The plates were incubated at 23 °C for 1 week. Strain T6T was isolated from 0.1× TSA plates, whereas T18T was isolated from a 0.01× TSA plate. The isolation of the strains was carried out by re-streaking on fresh media until a pure culture was obtained. Cell suspensions in TSA and R2A were cryopreserved at −80 °C with 15% glycerol (v/v). Their taxonomic status was determined by a polyphasic approach. On the basis of the results from phylogenetic, phenotypic and chemotaxonomic analysis, it is concluded that strains T6T and T18T are related to members of the genus Belnapia and representatives of two novel species. In the present work,
the reference strains *B. rosea* DSM 23312<sup>T</sup>, *B. moabensis* DSM 16746<sup>T</sup> and *B. soli* DSM 28067<sup>T</sup>, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and strains T6<sup>T</sup> and T18<sup>T</sup> were all grown in parallel on R2A media at 30 °C, unless otherwise specified.

The phenotypic characteristics of T6<sup>T</sup> and T18<sup>T</sup> were analysed after 1 week of growth at 30 °C. A Gram staining test was carried out with KOH 3 % (w/v), recording viscosity as a negative result. Oxidase activity was tested by using the commercial Oxidase Test Stick for microbiology (PanReac AppliChem). Catalase activity was tested with hydrogen peroxide 30% (v/v), recording bubble formation as a positive result. Cell morphology was observed under an optical microscope with crystal violet glass stain. Growth at different temperatures (4, 10, 15, 20, 23, 30, 37, 40, and 45 °C) and NaCl concentrations (0.0–4.0% at intervals of 0.5%) was checked on R2A. Growth at different pH values (4.0–10.0 at intervals of 1.0 pH unit) was examined by growing the strains in liquid R2A using the buffers MES (pH 4–6), HEPES (pH 7–8) and CHES (pH 9–10) at a final concentration of 10 mM. Growth under microaerophilic and anaerobic conditions was tested by incubating the plates in a candle jar and with the BD GasPak EZ pouch system (Becton, Dickinson), respectively. Carbon source assimilation and enzymatic activities were checked using the API 20NE and API ZYM system strips (bioMérieux) according to manufacturer's instructions. BIOLOG GEN III MicroPlates (BIOLOG) were also used to determine carbon source assimilation.

Strains T6<sup>T</sup>, T18<sup>T</sup> and the reference strains *B. rosea* DSM 23312<sup>T</sup>, *B. moabensis* DSM 16746<sup>T</sup> and *B. soli* DSM 28067<sup>T</sup> were grown on R2A medium at 30 °C for 72 h for analysis of cellular fatty acids. The analysis was carried out following the protocol recommended by MIDI Microbial Identification System (version 6.1, MIDI, Inc, Newark, DE, USA) [5]. The fatty acids content was analysed on a 6850 gas chromatography system (Agilent) using the TSBAs6 method [6].

Genomic DNA extraction was carried out using the DNeasy Power Soil kit (Qiagen), according to the manufacturer’s instructions, but incubating at 65 °C after the addition of C1. Whole 16S rRNA gene PCR was carried out with universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) [7] and 1492R (5′-GGTTACCTTGTAGCAG-3′) [8] following procedures described previously [4]. Phylogenetic trees based on the 16S rRNA gene sequences were reconstructed using the maximum-likelihood (ML) [9] and neighbour-joining (NJ) [10] methods with the software MEGA X v.10.1.7. The TamuraNei G+I evolutionary model and the Kimura two-parameter model were used for the ML and NJ trees, respectively. The reliability of the branch patterns was assessed using bootstrap analysis based on 500 and 1000 replicates, respectively, for the ML and the NJ trees [11].

The draft genome of strains T6<sup>T</sup> and T18<sup>T</sup> were sequenced with the NovaSeq 6000 system (Illumina; 2×150 bp paired-end sequencing). The genomic DNA was randomly fragmented by sonication, then DNA fragments were end polished, A-tailed and ligated with the full-length adapters for Illumina sequencing. Further PCR amplification was carried out with P5 and indexed P7 oligonucleotides, and PCR products for the final construction of the libraries were purified with an AMPure XP system. Libraries were then checked for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies), and quantified by real-time PCR. The FastQC tool (v0.11.5) [12] was utilized to assess the quality of the sequence reads. There were 14 375 848 and 12 879 132 paired-end reads for strains T6<sup>T</sup> and T18<sup>T</sup>, respectively before filtering. After quality filtering, there were considered to be 13 106 155 and 11 792 897 paired-end sequences the genomes of strains T6<sup>T</sup> and T18<sup>T</sup>, respectively. Genome assembly of paired reads was performed using the ‘--isolate’ mode in SPAdes (3.14.1) [13]. Assembly statistics were calculated with QUAST (v.5.0.2) [14] and the completeness and contamination levels were evaluated with CheckM (v.1.1.3) [15]. The draft genomes were annotated using the RAST tool kit (RAStk) [16] integrated in PATRIC v.3.6.8. The draft genomes were analysed with the TYGS tool [17] in order to identify the most closely related type strains to T6<sup>T</sup> and T18<sup>T</sup> with publicly available genomes and to calculate digital DNA–DNA hybridization (dDDH) indexes. JSpecies [18] was used for calculating the average nucleotide identities according to blast (ANIb) between genome pairs. UBCG (v.3.0) [19] was used for reconstructing the phylogenomic tree among the selected strains based on a multiple alignment of a set of 92 housekeeping genes. We selected the alignment method codon and inferred the phylogenetic relationships with FastTree. The reliability of the branch patterns was assessed using bootstrap analysis based on 100 replicates.

Strains T6<sup>T</sup> and T18<sup>T</sup> were aerobic, Gram-staining-negative, non-motile and coccus-shaped (0.8–1.0 µm in diameter). The cells of both strains occurred singly, as in other members of the genus *Belnapia*. Colonies were pink, irregular and mucous. T6<sup>T</sup> colonies were paler than those of the rest of the members of the genus *Belnapia*. After 3–5 days of growth at 30 °C, the colonies of both strains displayed a diameter of around 3–4 mm.

Both strains were able to grow at between 4 and 40 °C (optimum at 30 °C). Moreover, T6<sup>T</sup> was able to grow at up to 42 °C. Both strains showed tolerance to up to 1.5% (w/v) of NaCl (optimum 0–1%). *B. moabensis* DSM 16746<sup>T</sup> and *B. rosea* DSM 23312<sup>T</sup> showed similar NaCl tolerances, in contrast with *B. soli* DSM 28067<sup>T</sup>, which was able to grow at concentrations of up to 3%. All five strains were able to grow at between pH 5 and 9, with an optimum at 6–7 (Table 1).

Strains T6<sup>T</sup> and T18<sup>T</sup> showed, like the other members of the genus *Belnapia*, a positive response for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. In contrast, T6<sup>T</sup> and T18<sup>T</sup> and their relatives of the genus *Belnapia* showed a negative response for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotripsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-manosidase, α-fucosidase, fermentation
of glucose, arginine dihydrolysis and aesculin hydrolysis. B. moabensis DSM 16746T showed positive response for trypsin and gelatin, while B. rosea DSM 23312T showed positive response for reduction of nitrate and indole production. T6T was negative for urease, in contrast to the other four strains, which were positive or weakly positive for this activity. In API 20 NE strips, T18T was not able to assimilate any of the saccharides tested, whereas T6T could grow weakly with potassium gluconate, adipic acid and malic acid (Table S1). Furthermore, the results of the BIOLOG GENIII plates indicated that strains T6T and T18T were only able to oxidize five and four out of the 71 carbon sources, respectively (Table S1, available in the online version of this article). In contrast, the reference strains B. moabensis DSM 16746T, B. rosea DSM 23312T and B. soli DSM 28067T were able to oxidize 41, 25 and 16 carbon sources, respectively. This indicates that the reference strains present a more polytrophic metabolism than strains T6T and T18T.

Almost complete 16S rRNA gene sequences were obtained. The 16S rRNA gene sequence lengths of strains T6T and T18T are 1392 (accession number MW583035) and 1383 bp (MW583036), respectively. According to the EzBioCloud database tool, the most closely related type strains of T6T were B. rosea CGMCC 1.10758T (98.11%), B. moabensis CP2C7 (97.38%) and B. soli PB-K8T (96.80%); whereas the closest relatives of T18T were B. moabensis CP2C7 (98.55%), B. soli PB-K8T (97.54%) and B. rosea CGMCC 1.10758T (97.40%). The type strains B. rosea DSM 23312T, B. moabensis DSM 16746T and B. soli DSM 28067T were, thus, selected as comparative reference strains, which were obtained from the DSMZ- German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany).

The phylogenetic positions of strains T6T and T18T within the genus Belnapia were confirmed by both 16S-rRNA-based ML and NJ phylogenetic trees (Figs 1 and S1). T6T grouped with B. rosea CGMCC 1.10758T, whereas T18T showed an external position in the cluster formed by Belnapia moabensis CP2C7 and Belnapia soli PB-K8T in both trees. This phylogenetic inference was supported by high bootstrap values.

| Characteristic | Strain | Strain | Strain | Strain | Strain |
|---------------|--------|--------|--------|--------|--------|
| Isolation source | Biocrust | Biocrust | Soil crust | Forest soil | Grass soil |
| Temperature range (°C) | 4–42 | 4–40 | 4–40 | 4–40 | 4–40 |
| pH range | 5–9 | 5–9 | 5–9 | 5–9 | 5–9 |
| NaCl tolerance (% | 0–1.5 | 0–1.5 | 0–2 | 0–1.5 | 0–3 |
| Carbon source utilization (API 20NE) | | | | | |
| d-glucose | – | – | – | – | w |
| l-arabinose | – | – | w | w | w |
| d-mannose | – | – | – | w | – |
| Potassium gluconate | w | – | – | w | – |
| Adipic acid | w | – | – | + | + |
| Malic acid | w | – | – | w | – |
| Enzymatic activity (API 20NE) | | | | | |
| Nitrate reduction | – | – | – | + | – |
| Indole production | – | – | – | + | – |
| Urease | – | w | + | w | w |
| Gelatin | – | – | + | – | – |
| Enzymatic activity (API ZYM) | | | | | |
| Trypsin | – | – | + | – | – |
The draft genomes of strains T6T and T18T consisted of 220 and 355 contigs, respectively, which constituted a total length of 6449681 and 6937094 bp, respectively. The N50 values were 328210 and 194160 for T6T and T18T respectively. The genomic DNA G+C contents were 69.80 and 68.96% for T6T and T18T respectively, which is in accordance with the values previously described for the rest of the species within the genus *Belnapia* and further confirms their adscription to this genus [1–3]. A total of 6369 and 7450 coding sequences (CDSs) were predicted for strains T6T and T18T, of which 3380 and 3480, respectively, corresponded to proteins with functional assignment. Regarding the prediction of tRNA and rRNAs, a total of 49 and 47 tRNAs, and 3 and 2 rRNAs were predicted for strains T6T and T18T, respectively. The 16S rRNA gene sequences of strains T6T and T18T were also extracted from the genome, which were 1496 and 1494 bp long, respectively (accession numbers MW960268 and MW960269, respectively).

In order to obtain a more accurate phylogenetic inference of novel strains, a phylogenomic tree based on nucleotide sequences was reconstructed (Fig. 2). The phylogenomic tree corroborated that the two strains represent members of the genus *Belnapia*. Strain T18T was most closely related to *B. moabensis* DSM 16746T, while T6T showed an external position to the rest of the members of the genus *Belnapia*. The type strain of *B. soli* was not included in this analysis because its genome was not publicly available at the time of writing.

The ANIb and digital DDH values between strains T6T and T18T and other related species were calculated (Table S2). The ANIb and dDDH values of strain T6T vs. *Belnapia rosea* CGMCC 110758T were 83.26 and 29 %, respectively; the ANIb and dDDH values of strain T18T vs. *Belnapia moabensis* DSM 16746T were 88.47 and 40.5 %, respectively. Moreover, both genome indexes were calculated between strains T6T and T18T, which were 82.96 and 28.5 % for ANIb and dDDH, respectively. As the values were below the thresholds established to circumscribe prokaryotic species, namely 95 % for ANI values [20] and 70 % for dDDH [21], both genome indexes confirmed the classification of strains T6T and T18T as representing novel species [22].
The analysis of the genome of strains T6T and T18T allowed the prediction of their ability to synthesize phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol on the basis of the presence of genes coding for phosphatidylglycerol phosphatase (EC 3.1.3.27), phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17) and cardiolipin synthase (EC 2.7.8.-) respectively. This polar lipids’ profile is in agreement with the polar lipid analyses results available for other species of the genus Belnapia [1–3]. Furthermore, their ability to synthesize phosphatidylethanolamine was predicted on the basis of the presence of the genes coding for phosphatidylserine synthase (EC 2.7.8.8) and phosphatidylserine decarboxylase (EC 4.1.1.65), which had been previously described for B. soli [3].

The major fatty acid for strains T6T and T18T was summed feature 8 (C_{18:1\omega7c}/C_{18:1\omega6c}) (41.4 and 51.5%, respectively). However, there were also high amounts of C_{16:0} (15.7 and 12.9%, respectively for T6T and T18T), C_{18:1\omega2-\text{OH}} (12.2 and 10.0%, respectively) and summed feature 3 (C_{16:1\omega7c}/C_{16:1\omega6c}) (10.3 and 12.2%, respectively) (Table 2). This is in accordance with the profiles obtained for the members of the genus Belnapia, which also showed high amounts of summed feature 8, summed feature 3, C_{18:1\omega2-\text{OH}} and C_{16:1\omega7c}/C_{16:1\omega6c}, thus confirming the inclusion of both strains within the genus Belnapia.

The results of the phenotypic, chemotaxonomic, genomic and phylogenetic analyses confirm that strains T6T and T18T should be considered as each representing a novel species within the genus Belnapia, for which the names Belnapia mucosa sp. nov. and Belnapia arida sp. nov., respectively, are proposed.

**DESCRIPTION OF BELNAPIA MUCOSA SP. NOV.**

*Belnapia mucosa* (mu.co’sa. L. fem. adj. mucosa, mucous, slimy).

Colonies are circular, smooth, mucous, convex and pale-pink. Cells are Gram-reaction-negative, coccoid (0.8–1.0 µm) and non-motile. Growth occurs at 4–42 °C (optimum at 30 °C) and pH 5–9 (optimum 6–7), and it can tolerate up to 1.5 % (w/v) NaCl (optimum 0–1%). This species grows under aerobic and microaerophilic conditions, no growth is observed under anaerobic conditions. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are detected. Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase,
Belnapia arida (a’ri.da. L. fem. adj. arida, dry, referring to the isolation of the strain from an arid soil)

Colonies are circular, smooth, mucous, convex and pink. Cells are Gram-negative, coccal-shaped (0.8–1.0 µm) and non-motile. Growth occurs at 4–40 °C (optimum at 30 °C) and pH 5–9 (optimum 6–7), and it can tolerate up to 1.5% (w/v) NaCl (optimum 0–1%). This species grows under aerobic and microaerophilic conditions. No growth is observed under anaerobic conditions. The type strain T6T (CECT 30228=DSM 112073T) was first isolated from the Tabernas Desert in Almería (Spain) from a biocrust sample. The DNA G+C content of the type strain is 69.80%. The DDBJ/ENA/GenBank accession number for the 16S rRNA gene sequence is MW960268 and the genome accession number is JAEUXJ00000000.

**DESCRIPTION OF BELNAPIA ARIDA SP. NOV.**

Belnapia arida (a’ri.da. L. fem. adj. arida, dry, referring to the isolation of the strain from an arid soil)
dextrin, lactose, d-mannose, d-mannitol, glycy1-l-proline, d-galacturonic acid, methyl pyruvate, γ-aminobutyric acid, maltose, melibiose, d-fructose, d-arabitol, l-alanine, l-galactonic acid lactone, d-lactic acid methyl ester, α-hydroxybutyric acid, trehalose, methyl β-D-glucoside, d-galactose, myo-inositol, l-arginine, l-lactic acid, cellobiose, d-salicin, 3-methyl-D-glucoside, glycerol, l-aspartic acid, d-glucuronic acid, citric acid, α-ketoglutaric acid, acetooactic acid, sucrose, N-acetyl-l-β-D-mannosamine, l-fucose, d-fructose-6-phosphate, l-glutamic acid, glucuronamide, α-ketoglutaric acid, acetoacetic acid, L-lysine, N-acetyl-D-galactosamine, L-rhamnose, d-aspartic acid, quinic acid, acetic acid, stachyose, N-acetyl neuraminic acid, inosine, D-serine, L-serine, D-saccharic acid, bromosuccinic acid and formic acid. The major fatty acids are summed feature 8 (C18:0;7c/C18:1ω6c), C16:0, summed feature 3 (C16:1ω7c/C16:1ω6c) and C18:1ω2-OH.

The type strain T18T (CECT 30229T = DSM 112074T) was first isolated from the Tabernas Desert in Almería (Spain) from a biocrust sample. The DNA G+C content of the type strain is 68.96%. The DDBJ/ENA/GeneBank accession number for the 16S rRNA gene sequence is MW960269 and the genome accession number is JAETWB000000000.

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**Author contributions**
E.M.M., sampling, experimental procedures, bioinformatic analysis, writing and approving the manuscript. A.V.V., experimental procedures, bioinformatic analysis, writing and approving the manuscript. L.S., experimental procedures, bioinformatic analysis, writing and approving the manuscript. A.C., experimental procedures, bioinformatic analysis, writing and approving the manuscript. J. Pascual, supervising experimental work, bioinformatic analysis, writing and approving the manuscript. J. Peretó, writing and approving the manuscript. M.P., conceived the work, sampling, writing and approving the manuscript.

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

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