Citrobacter portucalensis sp. nov., isolated from an aquatic sample

Teresa Gonçalves Ribeiro,† Bruno Ribeiro Gonçalves,‡ Mickael Santos da Silva, Ángela Novais,† Elisabete Machado,‡ João André Carriço‡ and Luísa Peixe†,*

Abstract

A Gram-stain-negative strain, A60T, isolated from a water well sample in Portugal, was characterized phenotypically, genotypically and phylogenetically. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain A60T belonged to the genus Citrobacter, and recN gene phylogeny revealed a strongly supported clade encompassing strain A60T and 13 other strains from public databases, distinct from currently recognized species of the genus Citrobacter. Furthermore, multilocus sequence analysis (MLSA) based on concatenated partial fusA, leuS, pyrG and rpoB sequences confirmed the classification obtained with the recN sequence. In silico genomic comparisons, including average nucleotide identity (ANI) and the genome-to-genome distance calculator (GGDC), showed 94.6% and 58.4% identity to the closest relative Citrobacter freundii ATCC 8090T, respectively. The ability to metabolize different compounds further discriminated strain A60T from other species of the genus Citrobacter. The G+C content of strain A60T is 52.0%. The results obtained support the description of a novel species within the genus Citrobacter, for which the name Citrobacter portucalensis sp. nov. is proposed, with the type strain A60T (=DSM 104542T=CECT 9236T).

The genus Citrobacter comprises 13 species recognized by the International Committee on Systematics of Prokaryotes, including two species recently described: Citrobacter pasteurii and Citrobacter europaeus [1, 2]. Members of this genus are part of the normal intestinal flora of humans and animals, and can be isolated from a variety of environmental sources, although they also constitute important agents of opportunistic infections in humans [2, 3]. Moreover, some species of the genus Citrobacter have chromosomal antibiotic resistance genes (qnrB and blaCMY-2) which can be mobilized to mobile genetic elements [4, 5], (http://ardb.cbcb.umd.edu/) and/or have biotechnological potential [6, 7]. Nevertheless, differentiation of species of the genus Citrobacter based on conventional tests has been problematic, preventing the recognition of species with greater medical or industrial significance [8, 9]. We had previously demonstrated that phylogenetic analyses based on recN (DNA repair protein) gene sequences provide an accurate discrimination among species of the genus Citrobacter, and furthermore unveiled isolates not affiliated to any previously recognized species [2, 4]. This was the case of strain A60T, isolated from a water well sample collected in Portugal [2, 4]. The purpose of this work was to define the taxonomic position of this strain.

Strain A60T was isolated from a water well sample collected in Cantanhede city, Centre region of Portugal (2008). The water well sample (100 ml) was processed by a vacuum membrane filtration procedure. Individual filters were pre-enriched in Brain Heart Infusion (37°C/48 h), and the resulting enrichment (0.1 ml) was seeded in MacConkey (MAC) agar plates (37°C/24 h). Closely related Citrobacter freundii, Citrobacter braakii ATCC 51113T and C. europaeus 97T were used for comparative phenotypic analysis. Strains were maintained on tryptic soy agar (TSA; Sigma-Aldrich) for short-term storage and in tryptic soy broth (TSB; Sigma-Aldrich) supplemented with 20% (v/v) glycerol at −80°C for long-term storage. Gram staining was carried out through the use of the Gram Staining kit (bioMérieux). Oxidase activity was tested by using 1% (w/v) tetramethyl-p-phenylenediamine (Merck) and catalase activity was

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Abbreviations: ANI, average nucleotide identity; GGDC, genome-to-genome distance calculator; MLSA, multilocus sequence analysis.

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The GenBank/EMBL/DDJB project and sample accession numbers for the annotated genomic sequences and sequence reads of strain A60T are PRJNA3691027 and SAMN06214758, respectively.

One supplementary table and one supplementary figure are available with the online Supplementary Material.
Fig. 1. Neighbour-joining tree based on recN gene sequences showing the relationships between strain A60T and type strains of other species of the genus *Citrobacter*. Genetic distances were constructed using Kimura’s 2-parameter method. Bootstrap values obtained after 1000 replicates are given at the nodes, and only values >90% are shown. Type strains of species of the genus *Citrobacter* are shown in bold and underlined, and the corresponding GenBank/Patric accession numbers are the following: JMTA01000005 (C. freundii ATCC 8090T), KF057886 (C. braakii CIP 104554T), KR998020 (C. portucalensis A60T), BBMW01000018 (C. werkmanii NBRC 105721T), FLYB01000007 (C. europaeus 97/79T), CDHL01000055 (C. pasteurii CIP 55.13T), KF057888 (C. youngae CIP 105016T), KF057887 (C. Rodentium Antwerp 2011T), CIP 106783T (C. amalonaticus).
evaluated in the presence of 3 % (v/v) aqueous hydrogen peroxide solution. Growth at different NaCl concentrations [0, 3.0, 6.0, 9.0, 12.0 and 15.0 % (w/v)] and temperatures (5, 10, 15, 20, 25, 30, 37, 50, 65 and 70 °C) were examined by using TSB as the basal medium. To determine the pH range for growth, basal medium was adjusted with HCl or NaOH to reach pH values of 4.0–11.0, at intervals of 1.0 pH unit. To confirm the ability of anaerobic growth, strains were inoculated into TSB tubes with paraffin on top. Biochemical characterization was performed using the standardized API 20E strips (incubation at 37 °C for 24 h) and API 50 CH strips (incubation at 37 °C for 48 h) (bioMérieux). Whole genome shotgun sequencing of strain A60 was achieved using Illumina MiSeq 2 × 250 nt. The draft genome of strain A60 was obtained using INNUca-INNUENDO Reads Control and Assembly (https://github.com/INNUENDOCON/INNUca), which provides a pipeline to check for read quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by de novo assembly with SPADes [10]. Annotation of the draft genome was performed using Prokka software [11]. In silico genome-to-genome comparison was assessed by using nucleotide identity (ANI) calculated by using both in-house scripts (https://github.com/bfrgoncalves/ANI_Calculator) and jSpecies [12], and by using the genome-to-genome distance calculator (GGDC 2.0) under the recommended Formula 2 (http://ggdc.dsmz.de/distcalc2.php) [13]. Partial nucleotide sequences of the housekeeping genes recN, rrs (16S ribosomal RNA), rpoB (RNA polymerase beta subunit), pyrG (CTP synthetase), fusA (elongation factor-G), and leuS (tRNA synthetase), with the latter four included in the multilocus sequence analysis (MLSA) scheme described by Clermont et al. [1], were aligned and the similarity scores were generated using MEGA software version 5.2.2 (http://www.megasoftware.net/) [14]. Phylogenetic trees were reconstructed using the neighbour-joining method [15]. In addition, genetic distances were estimated using Kimura’s 2-parameter model [16]. The reliability of internal branches was assessed from bootstrapping based on 1000 resamplings [17].

16S rRNA gene sequence variation provides limited resolution to discriminate among closely related species of the genus Citrobacter [1, 2]. Indeed, phylogenetic analysis based on 16S rRNA gene sequences showed that strain A60 falls in the previously recognized group I described by Warren et al. [18], along with type strains of C. freundii, Citrobacter youngae, C. braakii, Citrobacter werkmanii, Citrobacter gillenii, Citrobacter muriniae, C. pasteurii and C. europaeeus (98.9% to 99.9% identity) (Fig. S1, available in the online Supplementary Material).

The phylogenetic analysis based on recN showed that A60 and other strains for which recN sequences are currently available in public databases were grouped together and shared high similarity (99.1%), which was statistically supported by a bootstrap value greater than 95% (Fig. 1, Table S1). Furthermore, Fig. 1 clearly delineates strain A60 and closely related strains in a separate clade, which was 93.1% similar to C. freundii. Similar results were observed with the application of the MLSA scheme (fusA, leuS, pyrG and rpoB; 2082 nt), with strain A60 and eight strains with publicly available genome sequences representing a well-separated lineage supported by a bootstrap value of 100% (data not shown). Additionally, affiliation of intrinsic genes (qnrB and blaCMY-2) shows allele variation specific to each species of the genus Citrobacter (e.g. qnrB cluster I associated with Citrobacter sp. 1, which included A60) (data not shown), corroborating the definition of C. portucalensis as a novel species.

The ANI values of strain A60 compared with the type strains of C. braakii (92.6%), C. europaeeus (93.1%) and even C. freundii (94.6%) were below the species cut-off level of 95%. ANI values close to the proposed threshold for species delineation were also observed for two other closely related species (C. pasteurii CIP 55.13 and C. youngae CIP 105016; ANI=94.7 %) [1]. In addition, the intergenomic distance between strain A60 and the closest relative type strain of C. freundii presented a GGDC value of 58.4%, which is clearly below the proposed criterion for bacterial species (70%) and subspecies (79%) delineation [19], further supporting that at the whole genome level, strain A60 represents a novel species.

The novel isolate stained as Gram-negative. The rod-shaped cells (1–2 µm in diameter and 4–5 µm in length) were motile. Growth occurred at 20, 25, 30, 37 and 50 °C, and in the range of 0–15 % (w/v) NaCl and pH 5.0–10.0. Strain A60 demonstrated differential biochemical profiles compared to type strains of closely related species of the genus Citrobacter, which are summarized in Table 1.

Overall, phylogenetic analysis of different genotypic markers, genome comparisons, and the phenotypic behaviour strongly suggests that strain A60 represents a novel species within the genus Citrobacter, for which the name Citrobacter portucalensis sp. nov. is proposed.

**DESCRIPTION OF CITROBACTER PORTUCALENSIS SP. NOV.**

Citrobacter portucalensis (por.tu.cal.en’sis. N.L. masc. adj. portucalensis referring to Portugal, from where the bacterium was isolated).

Colonies are translucent and bright, and cells are Gram-stain-negative, rod-shaped (1–2 µm in diameter, 4–5 µm in
Plasmid length, motile and non-spore-forming. Facultatively anaerobic. Catalase- and oxidase-negative. Does not decompose gelatin. Voges-Proskauer test and indole production test are negative. The methyl red test is positive. Growth occurs in the range of 0–15 % (w/v) NaCl and at pH 5.0–10.0 in TSB. Produces H₂S and reduces nitrate to nitrite, but N₂ production is negative. Uses citrate as a carbon source. L-Arginine, L-lysine and L-tryptophan are not utilized. Urease activity is positive. Acid is produced from glycerol, L-arabinose, D-ribose, D-xylene, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbosé, L-rhamnose, inositol, D-mannitol, D-sorbitol, N-acetylglucosamine, arbutin, salicin, cellubiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, gentiobiose, L-fucose, potassium gluconate, 2-ketoglactonate and 5-ketoglactonate, but not from erythritol, D-arabinose, L-xylene, D-adonitol, methyl β-D-xlyopyranoside, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, aesculin ferric citrate, inulin, melezitose, starch, glycerogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol and L-arabitol.

The type strain is A60T (=DSM 104542T=CECT 9236T), isolated from a water well in Portugal. The DNA G+C content of the type strain is 52.0%. Strains of C. portucalensis have been isolated from aquatic samples (water well, fountain, borehole), feed, catheter tip and human urine in different countries, some cases associated with human disease (Table S1).

Table 1. Differential metabolic characteristics of strain A60T and closely related type strains of species of the genus Citrobacter

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Amino acid utilization</td>
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<tr>
<td>L-Arginine</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>L-Ornithine</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Acid production from:</td>
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<tr>
<td>L-Sorbose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Dulcitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Inositol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Arbutin</td>
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<td>Salicin</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Sucrose</td>
<td>–</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>–</td>
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<td>Gentibiose</td>
<td>+</td>
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<td>+</td>
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<td>D-Lylose</td>
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<td>+</td>
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<tr>
<td>Potassium 5-ketogluconate</td>
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Conflicts of interest
The authors declare that there are no conflicts of interest.

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