Description of *Citrobacter cronae* sp. nov., isolated from human rectal swabs and stool samples

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**TAXONOMIC DESCRIPTION**

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The GenBank accession numbers of the whole genome sequencing data for *Citrobacter cronae* Tue2-1T are VOSQ00000000 and MN548424 for 16S rRNA sequence, respectively.

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

**Abstract**

Nine independent Gram-negative bacterial strains were isolated from rectal swabs or stool samples of immunocompromised patients from two different wards of a university hospital. All isolates were phylogenetically analysed based on their 16S rRNA gene sequence, housekeeping gene *recN*, multilocus sequence analysis of concatenated partial *fusA*, *leuS*, *pyrG* and *rpoB* sequences, and by whole genome sequencing data. The analysed strains of the new species cluster together and form a separate branch with *Citrobacter werkmanii* NBRC105721T as the most closely related species. An average nucleotide identity value of 95.9–96% and computation of digital DNA–DNA hybridization values separate the new species from all other type strains of the genus *Citrobacter*. Biochemical characteristics further delimit the isolates from closely related *Citrobacter* type strains. As a result of the described data, a new *Citrobacter* species is introduced, for which the name *Citrobacter cronae* sp. nov. is proposed. The type strain is Tue2-1T with a G+C DNA content of 52.2 mol%.

**ISOLATION AND ECOLOGY**

*Citrobacter* species are Gram-negative micro-organisms frequently encountered in the environment, but also from the intestinal tract of humans [1–3]. They were also reported as opportunistic pathogens causing wound infections, abscesses, severe forms of meningitis, endocarditis or bloodstream infections [4–8].

In clinical microbiology laboratories, *Citrobacter* species represent up to 6% of all isolated *Enterobacteriaceae* from clinical specimens [4]. As they can have chromosomal AmpC β-lactamases [9] as well as plasmid encoded carbapenemases [10], many antibiotics are ineffective increasing the intricacy of treatment [11]. To date, 15 *Citrobacter* species are published in the literature [12]. In the present study, nine independent clinical isolates (Tue2-1T, Tue2-3 and Tue2-5–Tue2-11) were investigated that originated from nine patients of two different wards with underlying haematological conditions. Isolates were collected and stored from rectal swabs or stool samples over a 3-year period (2012–2015), but even more isolates were obtained since 2016. Three of the strains (Tue2-1T, Tue2-3, Tue2-5) harbouring metallo-β-lactamase (MBL) enzymes were already characterized by comparative genomics using next generation sequencing, but could not be unambiguously identified to the species level by standard routine methods [13]. We first considered that the studied isolates belong to the species *Citrobacter werkmanii*. However, experimental evidence suggested that the three isolates belong to a new *Citrobacter* species, which will be characterized here. In order to gain further evidence for our new hypothesis we additionally characterized and sequenced six more isolates (Tue2-6–Tue2-11).

**METHODS**

MALDI-TOF AXIMA system assurance (bioMérieux; Saramis database version 4.09) and the Microflex LT instrument (Bruker Daltonics; MBT IVD Library.5627) were
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performed on all isolates, but failed to unambiguously identify the strains. Additionally, biochemically based identification using the API 20 E System (bioMérieux; apiweb) and the vitek GN ID card (bioMérieux) was applied. Bacterial DNA was extracted from cultures grown on Columbia agar with 5% sheep blood (Becton Dickinson) using the Genomic-tip 100/G system (Qiagen) following the manufacturer’s instructions. For whole genome sequencing, libraries were prepared using the TruSeq DNA LT Sample Prep Kit (Illumina) with 24 different barcodes using standard protocols as described previously [13, 14]. Barcoded libraries were analysed by the Agilent 2100 Bioanalyzer (Agilent Technologies) or QIAxcel Advanced Instrument (Qiagen) and quantified by Real-Time (RT)-PCR. Normalized libraries were pooled and sequenced with v3 chemistry (2x300 bp) or with v2 chemistry (2x250 bp) on the MiSeq platform (Illumina). Assembly of genome sequences was performed using SPAdes (version 3.7.0) [15] with default settings.

For phylogenetic analysis of the isolates, publicly available whole genome sequencing (WGS) data from Citrobacter type strains were included in the analysis (Table S1). Progressive-Mauve (version 2.3.1) [16] was run to conduct a full alignment of 23 genomes using default settings and prophage regions were investigated and excluded using phaster (phaster.ca) [17]. Maximum-likelihood phylogenetic trees of 23 whole genome-sequences were reconstructed by applying IQ-Tree with 1000 bootstrap replicates. Alignments of 16S rRNA gene sequences downloaded from EZ-Taxon [18], concatenated partial fusA (protein synthesis elongation factor-G), leuS (leucine tRNA synthetase), pyrG (CTP synthetase) and rpoB (β-subunit of RNA polymerase) [19] as well as recN.
sequences (DNA repair) [2, 20] used for recent description of new Citrobacter species extracted from available WGS data were done by clustal_w (BioEdit version 7.2.5) followed by phylogenetic treeing with RAxML and the GTR model in conjunction with GAMMA rates [21]. Trees were visualized using FigTree (version 1.4.3). The average nucleotide identity (ANI) was assessed by JSpecies (version 1.2) [22, 23] based on BLAST +2.2.29 (ANIB). The Genome-to-Genome Distance Calculator (GGDC 2.1) using the recommended Formula 2 was applied for in silico genome comparison and computation of digital DNA–DNA hybridization (dDDH) values [24]. Employing the online multi-locus sequence typing (MLST) service of the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST/; version 2.0), MLST sequence types were obtained from assembled sequences based on the MLST scheme for C. freundii [25].

**PHYLOGENY**

MALDI-TOF using Bruker and bioMerieux systems as well as analysing the biochemical characteristics of these strains with API 20E and VITEK2 system (bioMerieux) did not allow for unambiguous identification of all nine study isolates on the species level. Dissecting the MLST type showed the same result for all nine strains isolated over the 3-year collection period. Their phylogenetic relationship to other Citrobacter type strains was assessed by analysing the 16S rRNA, the recN gene, the concatenated partial fusA, leuS, pyrG and rpoB genes as well as by WGS. 16S rRNA gene-based phylogeny represented a distinct branch of all isolates of the new Citrobacter species clustering together in group I including the formerly published species Citrobacter freundii, Citrobacter youngae, Citrobacter braakii, Citrobacter werkmanii, Citrobacter gillenii and Citrobacter murliniae [26] as well as Citrobacter pasteuri [19] and the recently described C. europaeus [2] and C. portu-calensis [20] (Fig. S1, available in the online version of this article). Regarding the limited resolution of 16S rRNA genes in discrimination of Citrobacter species [19, 27], the closest similarity in 16S rRNA gene comparison was found to C. freundii (99.73%). Phylogenetic analysis based on the recN gene (Fig. S2) as well as MLSA of concatenated partial fusA, leuS, pyrG and rpoB (Fig. 1) extracted from WGS data of type strains confirmed 16S rRNA gene-based clustering of all nine isolates in a separate branch. The maximum-likelihood tree generated using WGS data enabled further distinction of the new Citrobacter species from other type strains of the genus including the most closely related species C. werkmanii strain NBRC 105721T (Fig. 2).
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**GENOME FEATURES**

Species definition can also be based on ANI value [22, 28]. Therefore the new Citrobacter isolates were compared to all Citrobacter type strains with available WGS data (Table S2b). The closest relationship of the new Citrobacter species was found with C. werkmanii NBRC105721T (95.92%), slightly below the proposed cut-off value of 96% for the assignment of a new species [29]. In comparison, ANI values between all nine analysed isolates (Tue2-1, Tue2-3, Tue2-5–2–11) were above 99.5%, demonstrating their close relationship (Table S2a).

As described recently, dDDH can be used for delineation of a new bacterial species using WGS data [29]. As illustrated in Table S2b, dDDH values were calculated for all available Citrobacter type strains in relation to Tue2-1T. The lowest intergenomic distance of our nine analysed Citrobacter species isolates was found to C. werkmanii NBRC105721T with a dDDH value of 70%, exactly the cutoff proposed for bacterial species delineation [22, 28]. The dDDH values between any pair of the new Citrobacter species isolates were above 99.1%.

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**Table 1. Biochemical characteristics of all Citrobacter cronae study isolates and closely related Citrobacter type strains**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<th>6</th>
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<th>8</th>
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<td>+</td>
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<td>+</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>−</td>
<td>v</td>
<td>+</td>
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<td>Phosphatase</td>
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<td>+</td>
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<td>NA</td>
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<td>NA</td>
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<tr>
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<td>−</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>−</td>
<td>NA</td>
<td>v</td>
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<td>v</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>β-Glucosidase</td>
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<td>−</td>
<td>v</td>
<td>−</td>
<td>v</td>
<td>v</td>
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<tr>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>H₂S</td>
<td>v (44.4)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td></td>
</tr>
<tr>
<td>Malonate</td>
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<td>−</td>
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<td>+</td>
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<tr>
<td>Ornithine</td>
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<td>−</td>
<td>−</td>
<td>v</td>
<td>−</td>
<td>NA</td>
<td>−</td>
<td>NA</td>
<td>v</td>
</tr>
<tr>
<td>Sucrose</td>
<td>v (77.7)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>v</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5-Ketogluconate</td>
<td>v (66.6)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Data obtained from  This study  This study  [19, 30]  [19, 30]  [19]  [20]  [19, 30]  [2]  [19, 30]
PHYSIOLOGY

Biochemical characteristics were analysed by the API 20E and VITEK2 systems and results are listed in Table 1 for all nine study isolates and closely related type strains of the genus *Citrobacter* [2, 19, 20, 30]. The data show that all nine C. *cronae* isolates are able to catalyse amylase, catalase, deaminase, distinguishing the new species from all other closely related *Citrobacter* species tested. In addition all *C. cronae* isolates are able to catalyse cellobiose, which is not the case for C. *werkmanii DSM17579*. No enzymatic activity for phosphatase could be found for *C. cronae*, whereas C. *werkmanii DSM17579* was positive for phosphatase. Moreover due to some variable characteristics not found to be diverse in different C. *werkmanii* isolates [19, 30], C. *cronae* can be separated biochemically. Taken together, phylogenetic analysis based on 16S rRNA gene, *recN*, the concatenated partial genes *fusA*, *leuS*, *pyrG* and *rpoB* and WGS data, calculation of genome relatedness by ANI and DDDH as well as biochemical properties classifies *Citrobacter* Tue2-1, 2-3 and 2-5-2-11 as representing a new species within the genus *Citrobacter* for which we propose the name *Citrobacter cronae* sp. nov., with Tue2-1T as type strain.

DESCRIPTION OF CITROBACTER CRONAE SP. NOV.

*Citrobacter cronae* [cro’nae. N.L. gen. n. cronae, pertaining to the CRONA (the landmark building of the university hospital; acronym for Surgery, Radiology, Orthopedics, Neurology, Anesthesiology) clinics, Tuebingen, Germany].

*Citrobacter cronae* is a Gram-stain-negative, oxidase-negative, catalase-positive (delayed), facultative anaerobic, rod-shaped bacterium. It is able to ferment the following carbohydrates: d-mannitol, sorbitol, d-mannose, cellobiose, trehalose and amygdalin. The strains cannot utilize aesculin, inositol and melibiose and are negative for acetoine- and indole production. Variable reactions are observed for fermentation of d-glucose 1-rhamnose, arabinose, maltose, maltolone, d-adonitol, citrate, potassium 5-keto-gluconate, sucrose and d-tagatose. *Citrobacter cronae* is variable for urease and production of H₂S.


The type strain of *Citrobacter cronae* is Tue2-1T, which was isolated from a rectal swab of a patient hospitalized at University Hospital Tuebingen, Tuebingen, Germany. The G+C DNA content of the type strain is 52.2 mol%. The accession numbers for the WGS and 16S rRNA gene of strain Tue2-1T are VOSQ0000000 and MN548424, respectively. The culture certificate accession numbers are CCUG 73860 from the CCUG, Göteborg, Sweden, and DSM 110040 from the DSMZ, Braunschweig, Germany.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The study was conducted in accordance with the local ethics committee of the medical faculty of the university clinics at Tübingen, Germany (407/2013R).

References


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