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TAXONOMIC DESCRIPTION

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

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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* NBRC105721^T 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 Tue2-1^T 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 (Tue2-1^T, 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 (Tue2-1^T, 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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Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization; MLSA, multilocus sequence analysis; MLST, multilocus sequence typing.

The GenBank accession numbers of the whole genome sequencing data for *Citrobacter cronae* Tue2-1^T 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.

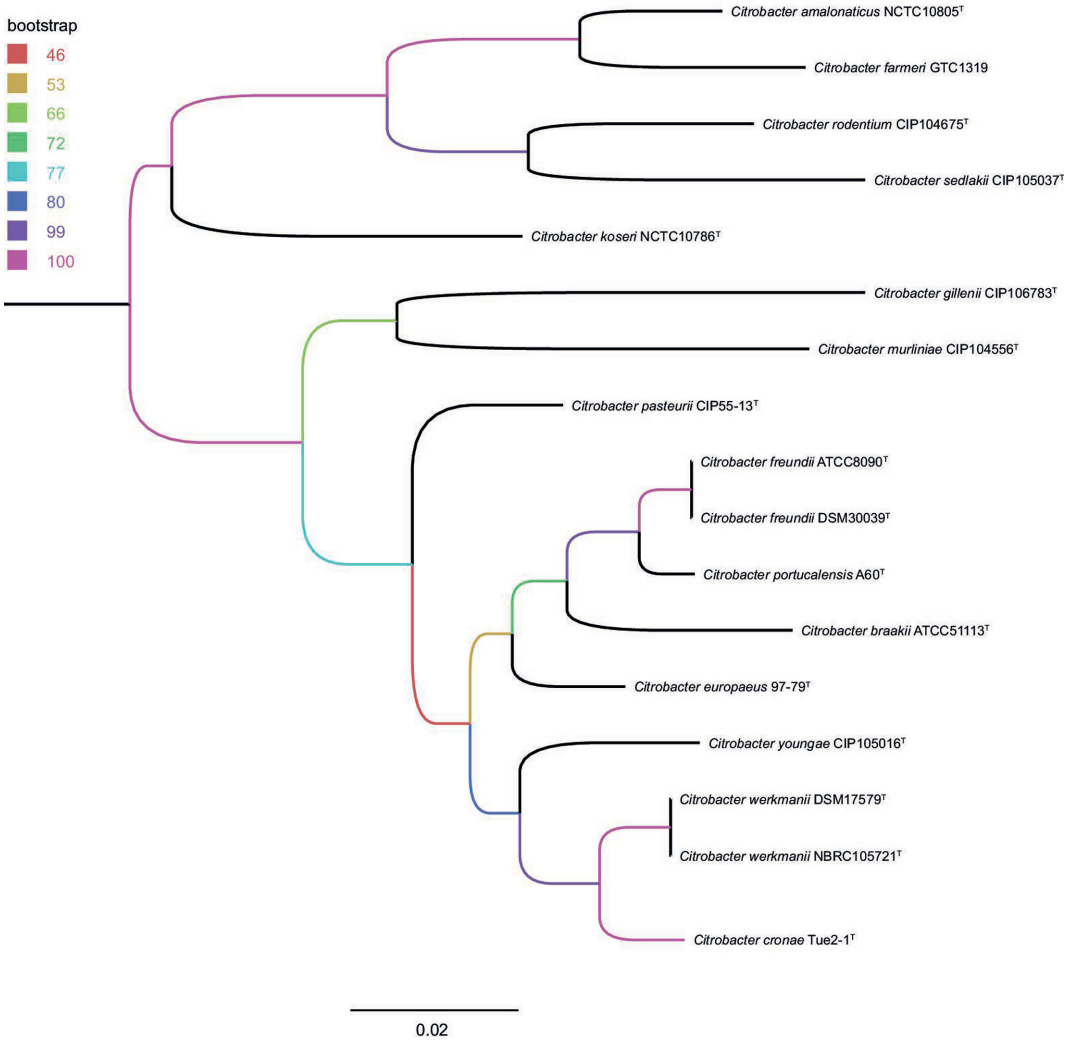

Fig. 1. Multilocus sequence analysis of concatenated partial *fusA*, *leuS*, *pyrG* and *rpoB* gene sequences extracted from whole genome data of the study isolates (*Citrobacter cronae* Tue2-1^T, Tue2-3, Tue2-5 – Tue2-11) and available genome data of *Citrobacter* type strains. The scale bar represents the expected number of changes per site. Bootstrap values [%] are colour-coded for all nodes (based on 1000 replicates). The tree was rooted at midpoint.

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 (2×300 bp) or with v2 chemistry (2×250 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, publically 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*

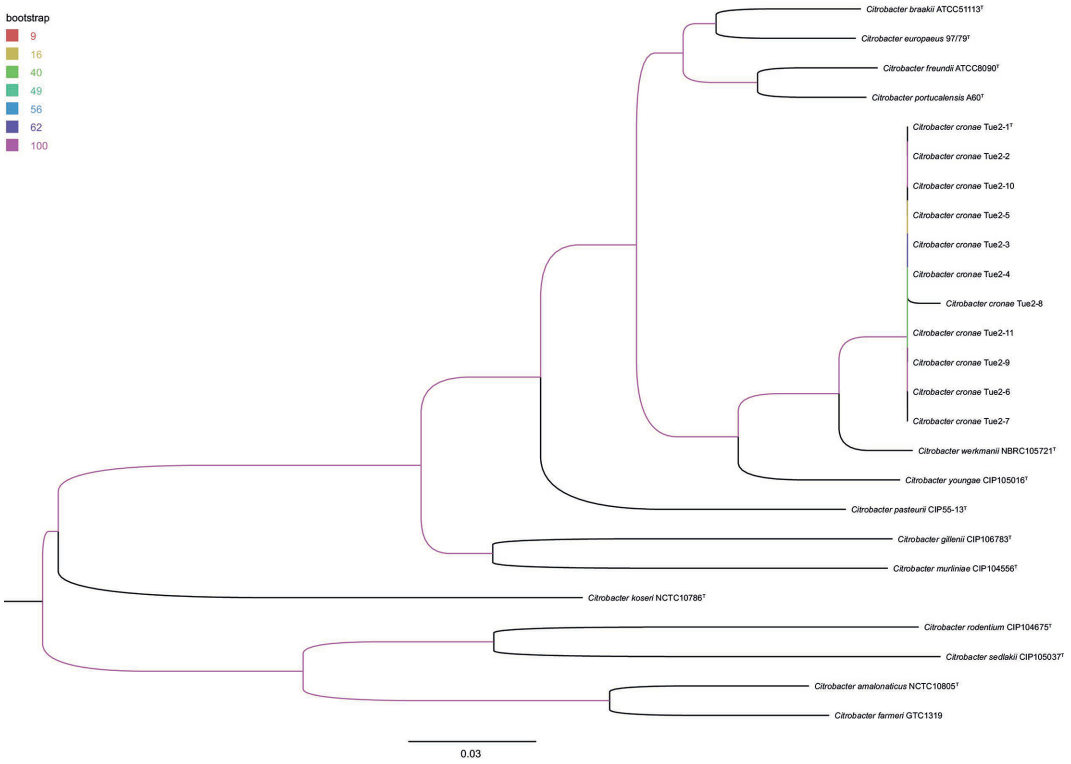

Fig. 2. Maximum-likelihood phylogeny based on whole genome sequencing data of the study isolates (*Citrobacter cronae* Tue2-1^T, Tue2-3, Tue2-5 – Tue2-11) and available genome data of *Citrobacter* type strains. The scale bar represents the expected number of changes per site. Bootstrap values (%) are colour-coded for all nodes (based on 1000 replicates). The tree was rooted at midpoint.

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 bioMérieux systems as well as analysing the biochemical characteristics of these strains with API 20E and VITEK2 system (bioMérieux) 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 pasteurii [19] and the recently described *C. europaeus* [2] and *C. portucalensis* [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 105721^T (Fig. 2).

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

Strains: 1, Tue2-1^T–Tue2_11; 2, *Citrobacter werkmanii* DSM17579^T; 3, *Citrobacter werkmanii*; 4, *Citrobacter youngae*; 5, *Citrobacter pasteurii*; 6, *Citrobacter portucalensis* A60^T; 7, *Citrobacter freundii*; 8, *Citrobacter europaeus* 97/79^T; 8, *Citrobacter braakii*. +, Positive result; –, negative result; v, variable result; NA, not available. For variable reactions of *Citrobacter cronae* isolates (n=9), values in parentheses represent percentage of positive strains.

Characteristics	1	2	3	4	5	6	7	8	9
Amygdalin	+	–	–	–	–	–	–	–	–
Cellobiose	+	–	v	v	+	+	v	+	v
Catalase	+	+	v	v	–	–	+	+	+
Phosphatase	–	+	NA	NA	+	NA	NA	NA	NA
α-Glucosidase	–	–	+	v	–	NA	v	NA	v
Indole	–	–	–	–	–	v	–	–	v
Melibiose	–	–	–	v	–	+	+	+	v
β-Glucosidase	v (66.6)	–	–	v	+	NA	v	NA	–
Adonitol	v (44.4)	–	–	–	–	–	–	–	–
H_2S	v (44.4)	+	+	+	+	+	+	+	v
Malonate	v (88.8)	+	+	–	–	NA	–	NA	+
Ornithine	v (44.4)	–	–	v	–	NA	–	NA	v
Sucrose	v (77.7)	–	–	–	v	+	+	–	–
5-Ketogluconate	v (66.6)	–	–	+	+	+	+	+	+
Data obtained from	This study	This study	[19, 30]	[19, 30]	[19]	[20]	[19, 30]	[2]	[19, 30]

MALDI-TOF using Bruker and bioMérieux systems as well as analysing the biochemical characteristics of these strains with API 20E and VITEK2 system (bioMérieux) 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 the recently described *C. europaeus* [2] and *C. portucalensis* [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 105721^T (Fig. 2).

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* NBRC105721^T (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 Tue2-1^T. The lowest intergenomic distance of our nine analysed *Citrobacter* species isolates was found to *C. werkmanii* NBRC105721^T 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%.

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 catabolize amygdalin distinguishing the novel species from all other closely related *Citrobacter* species tested. In addition all *C. cronae* isolates are able to catabolize cellobiose, which is not the case for *C. werkmanii* DSM17579^T. No enzymatic activity for phosphatase could be found for *C. cronae*, whereas *C. werkmanii* DSM17579^T 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^T, 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 Tue2-1^T as type strain.

DESCRIPTION OF *CITROBACTER CRONAE* SP. NOV.

Citrobacter cronae [cro'nae. N.L. gen. n. *cronae*, pertaining to he 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 and derivatives: D-mannitol, sorbitol, D-mannose, cellobiose, trehalose and amygdalin. The strains cannot utilize aesculin, inositol and melibiose and are negative for acetoin- and indole production. Variable reactions are observed for fermentation of D-glucose L-rhamnose, arabinose, maltose, malonate, D-adonitol, citrate, potassium 5-keto-gluconate, sucrose and D-tagatose. *Citrobacter cronae* is variable for urease and production of H_2S.

Semi-quantitative analysis of enzymatic activities of all strains demonstrate positive reactions for L-pyrrolidonyl-arylamidase and *β*-galactosidase, but negative reactions for α-glucosidase, phosphatase, lipase, lysine decarboxylase, tryptophan deaminase, gelatinase, α-galactosidase, Glu-Gly-Arg-arylamidase, *β*-*N*-acetyl-galactosaminidase, *β*-xylosidase, *β*-alanin-arylamidase-pNA and *β*-glucoronidase. Variable enzymatic reactions are seen for *N*-acetyl-*β*-glucosaminidase, ornithine decarboxylase, arginine dihydrolase, tyrosin arylamidase and *β*-glucosidase.

The type strain of *Citrobacter cronae* is Tue2-1^T, 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 Tue2-1^T are VOSQ00000000 and MN548424, respectively. The culture certificate accession numbers are CCUG 73860^T 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 from the medical faculty of the university clinics at Tübingen, Germany (407/2013R).

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