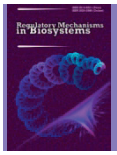

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Whole genome sequencing of the multidrug-resistant pathogen *Citrobacter werkmanii* recovered from a urinary tract infection patient (case in Mosul, Iraq)

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Citrobacter werkmanii is an emerging opportunistic human pathogen increasingly widespread in poor nations, responsible for wound, urinary tract, and bloodstream infections. The whole genome sequence of *C. werkmanii* SAS had a size of 5,072,546 bp and yielded a GC content of 51.99% distributed within 61 contigs; the largest contig was 941,705 bp, and the smallest was 528 bp with an N_{50} value of 351,029. Using the Rapid Annotation System Technology (RAST) server, 4793 coding sequences were detected in addition to 71 RNA genes from different categories. The phylogenetic taxonomy tree of *C. werkmanii* SAS, generated using the Type Strain Genome Server (TYGS), identified the closest type strains as *C. cronae* Tue2-1T (accession number: NZ_VOSQ01000380.1). A search for antibiotic resistance genes was conducted in the genome of *C. werkmanni* SAS utilizing the Comprehensive Antibiotic Resistance Database (CARD). The findings indicated that the genome harbored five key genes associated with resistance to various classes of antibiotics, such as fluoroquinolone, cephamycin, cephalosporin, glycopeptides, polypeptide antibiotics, and tetracyclines. A consistent GC content was noted in the SAS genome; however, regions with low GC content were identified at multiple locations. Those regions are an indicative of a possible horizontal gene transfer or insertions in such genomic locations.

Keywords: antibiotic resistance; *Citrobacter werkmanni*; whole genome sequencing.

Introduction

The genus *Citrobacter* consists of gram-negative, non-spore-forming bacilli that can use citrate as a carbon source, which belongs to the Enterobacteriaceae family (Jabeen et al., 2023). So far the genus *Citrobacter* has 18 species according to traditional methods and molecular approaches. *Citrobacter* species are widespread: they are found in the gastrointestinal tracts of humans and animals , and also in water, soil, and food (Forsythe et al., 2015). A range of transmission pathways have been put forward for this pathogen, including fecal-oral transmission, via contaminated food, through hospital equipment, as well as human-to-human transmission. *C. werkmanii*, though usually considered a commensal organism in human and animal species, has been previously found in wound infections, urinary tract infections, and bacteremia in humans (Aguirre-Sánchez et al., 2023).

Citrobacter koseri and *C. freundii* are the most common species most often associated with opportunistic human infections. Studies from North America showed that *Citrobacter* species were responsible for 3–6% of the hospital-acquired Enterobacteriaceae infections (Forsythe et al., 2015). *Citrobacter* has the ability to persist in a specific host for extended durations (Jabeen et al., 2023), leading to infections that may cause multidrug resistance outbreaks, particularly among neonates and immunocompromised patients with prolonged hospital stays (Thapa et al., 2009). Recent evidence has shown that other species belonging to the genus *Citrobacter*, including *C. werkmanii*, are recognized as emerging opportunistic pathogens in developing countries (Aguirre-Sánchez et al., 2023).

If antimicrobial resistance in bacteria rises, it will diminish the effectiveness of antibiotics, thereby restricting the therapeutic options available. The rising costs resulting from this situation could in certain instances prove untenable for worldwide health systems (Prestinaci et al., 2015; Abdulrazzaq et al., 2024). According to Jean et al. (2022), the increasing prevalence of carbapenemase-producing strains from Gram-negative Enterobacteriaceae, presents a huge challenge to patients health worldwide (Prestinaci et al., 2015; Peter et al., 2018). Enterobacteriaceae were the most common pathogenic bacteria in China's healthcare facilities in the first half of 2023, according to Chinet (www.chinets.com). Carbapenem resistance is making opportunistic infections harder to treat, which stresses the need for more investigation and research to understand the main mechanism of resistance (Ibrahim & Faisal, 2024).

Citrobacter species cause 3–6% of nosocomial infections caused by Enterobacteriaceae pathogens in North America (Forsythe et al., 2015) and most opportunistic infections in humans (Jabeen et al., 2023). Newborns and immunocompromised patients with long hospital stays are at risk of multidrug resistance epidemics. Even though *C. werkmannii* is less often researched than other *Citrobacter* species like *C. freundii*, this pathogen has become an opportunistic pathogen in humans. It has been linked to urinary tract infections, sepsis, wound infections, and meningitis in newborns among other diseases. In hospital environments or immunocompromised people, these infections especially raise issues. Moreover, *C. werkmanii* has been linked to multidrug resistance (MDR), therefore it might be a reservoir for genes conferring antimicrobial resistance. Analyzing its genome, will help to clarify processes of virulence, resistance, and transmission.

Materials and methods

Source and identification of Citrobacter werkmanii SAS. C. werkmanii SAS was isolated in a previous study from a UTI patient visiting Al-Hadbaa Teaching Hospital in Mosul, Iraq. The isolate produced smooth, lactose fermenting colonies on MacConkey agar. Identification was further confirmed via 16S rRNA gene sequencing.

Genomic DNA extraction and sequencing. Genomic DNA was gently extracted from *C. werkmanii* SAS using the Presto Mini Genomic DNA Bacterial Kit supplied by Geneaid (Taiwan). Genomic DNA was run on 2% agarose gel to confirm integrity (Fathi & Faisal, 2024) and sent for sequencing at Psomagene sequencing company (Maryland, USA).

Genome submissions to NCBI GenBank. The complete genome sequence of *C. werkmanii* SAS was deposited at DDBJ/ENA/GenBank under the accession number JBBWFL000000000.

Genome assembly and annotation. The raw reads underwent *de novo* assembly into contigs utilizing the SPAdes 3.5 bioinformatics

tool (Bankevich et al., 2012), with k-mer lengths set to 21, 33, 55, and 77. The assembly statistics were generated using QUAST software (Gurevich et al., 2013). The genome assembly underwent annotation through the RAST server (Aziz et al., 2008). The SEED tool was employed to predict functional genes within subsystem categories (Overbeek et al., 2014; Younis & Faisal, 2024).

Whole genome based phylogenetic tree. To construct a whole genome based phylogenetic tree for *C. werkmanii* SAS, along with their closely related strains, TYGS was employed (Meier-Kolthoff & Goker, 2019). A genome FASTA file was uploaded via the server using default parameters. The drawing of the phylogenetic tree was done using the FastME 2.0 as described elsewhere (Lefort et al., 2015).

In silico DNA-DNA hybridization analysis (isDDH). The bioinformatics tool, GGDH, was applied to obtain the isDDH values of *C. werkmanii* SAS based on comparison of its whole genome sequence information against its nearest related strains (Meier-Kolthoff et al., 2022).

16S rRNA gene phylogenetic tree analysis. To elucidate the homology of the *C. werkmanii* SAS sequence, a study of the sequence was carried out using the BLAST tool of NCBI. This was done by aligning the sequence with other sequences in the NCBI GenBank database. MEGA-11 software (Tamura et al., 2021) was used to construct the phylogenetic tree through bootstrap analysis (100X).

Detection of antibiotic resistance genes in the Genome of C. werkmanii SAS. Antibiotic resistance genes from *C. werkmanii* SAS genome was identified through the Comprehensive Antibiotic Resistance Database (CARD) program version 3.2.6 (Alcock et al., 2020; Ibraheem & Faisal, 2025).

Genome comparisons. The GView tool was applied to orient *C. werkmanii* SAS with the most relevant species, resulting an image which detects distinctions and parallels between the *C. werkmanii* SAS genome and other bacterial genome sequences as a series of concentric rings (Stothard et al., 2019).

Results

Table 1 shows the general genome features of *C. werkmanii* SAS, which clarifies that the overall size of the genome was 5,072,546 bp. The whole genome sequence of *C. werkmanii* SAS yielded a GC content of 51.99% distributed within 61 contigs; the largest contig was 941,705 bp, and the smallest was 528 bp with an N_{50} value of 351,029. Using the Rapid Annotation System Technology (RAST) server, we were able to detect 4793 coding sequences in addition to 71 RNA genes from different categories. Most of the genes predicted in the subsystem categories contribute to the metabolism of carbohydrates (403), amino acids and derivatives (381), protein metabolism (247), cofactors, vitamins, prosthetic groups, and pigments production (184), as indicated in Figure 1.

Table 1
General genome features of *Citrobacter werkmanii* SAS generated using QUAST software and RAST server

Feature	Value
Genome total length, bp	5,072,546
Number of contigs	61
Largest contig, bp	941,705
Smallest contig, bp	528
GC content, %	51.99
Total of protein-coding sequences (CDSs)	4,793
Number of tRNA genes	71
N_{50}	351,029

The phylogenetic taxonomy tree of *C. werkmanii* SAS, generated using the TYGS server, identified the closest type strains as *C. cronae* Tue2-1T (accession number: NZ_VOSQ01000380.1), *C. werkmanii* NBRC 105721 (accession number: NZ_BBMW00000000.1), *C. europaeus* 97/79 (accession number: NR_156052.1), *C. arsenatis* LY-1T (accession number: NZ_CP037864.1), *C. braakii* ATCC 51113 (accession number: NZ_NAEW00000000.1), with isDDH values of 83.4, 82.1, 71.4, 71.1, 67.7, respectively (Fig. 2 and Table 2).

The entire 16S rRNA gene of *C. werkmanii* SAS (shown in a black circle) was extracted from the sequenced genome and utilized to construct a phylogenetic tree. The phylogenetic tree was constructed utilizing MEGA-11 software, featuring a scale length of 0.01. The phylogenetic tree indicates that the *C. werkmanii* SAS strain is most closely related to *C. werkmanii* NBRC 105721, thereby confirming that the species isolated in our study is indeed a strain of *C. werkmanii*. Other species from *Citrobacter* were also found in various branches of the dendrogram, as illustrated in Figure 3 and Table 3.

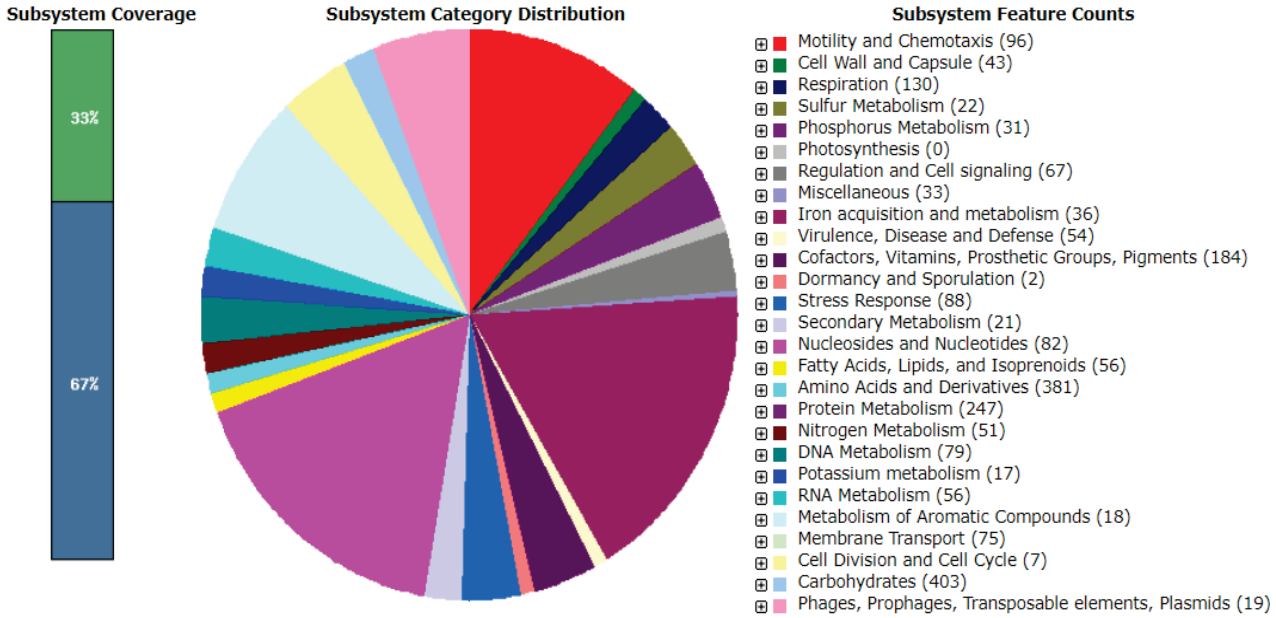

Fig. 1. Statistics on the distribution of subsystem categories for *Citrobacter werkmanii* SAS: the genome underwent annotation through the utilization of the Rapid Annotation System Technology (RAST) server; the pie chart illustrated the count of each subsystem feature, while the subsystem coverage was presented through the SEED viewer; the green bar of the subsystem coverage indicates the percentage of proteins that are part of the subsystems, while the blue bar represents the percentage of proteins that are not part of the subsystems

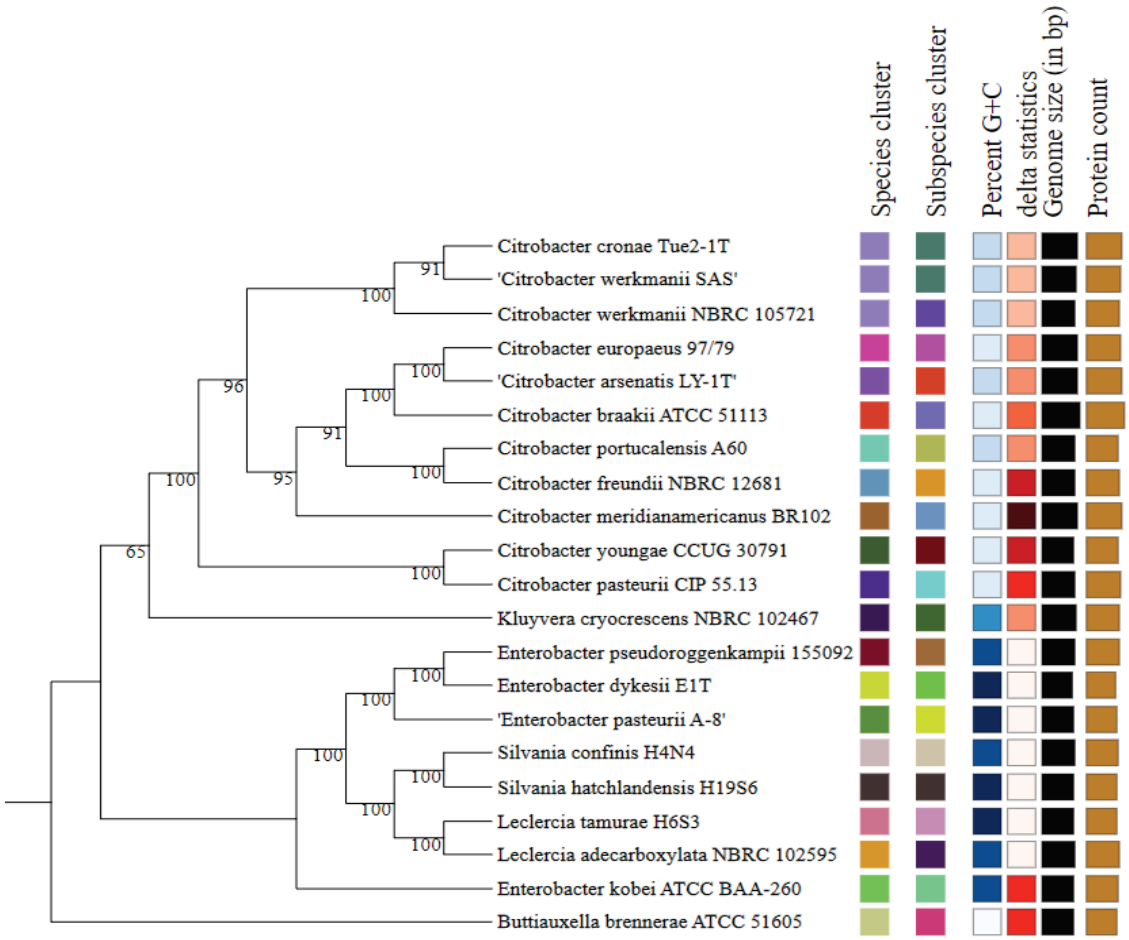

Fig. 2. Phylogenetic taxonomy tree of *Citrobacter werkmanii* SAS generated using the TYGS server: the final tree was constructed using the FastME 2.0 approach, which is grounded in the balanced minimum evolution method, incorporating 100X pseudo-bootstrap support values; labels on leaves are specified by their connection to species and subspecies clusters, genomic GC content, δ-values, overall genome size, and the total count of proteins

Table 2
Genome pairwise comparisons of *Citrobacter werkmanii* SAS genome vs. type strain genomes depending on isDDH, GC content, δ-value, genome size, and number of proteins

Citrobacter werkmanii SAS vs. other type strain genomes	Digital *is* DDH value, %	G+C, %	δ- value	Genome size, bp	Number of proteins
Citrobacter cronae Tue2-1T	83.4	52.41	0.119	5,210,578	5,025
Citrobacter werkmanii NBRC 105721	82.1	51.99	0.118	5,073,247	4,774
Citrobacter europaeus 97-79	71.4	51.89	0.128	5,283,764	4,896
Citrobacter arsenatis LY-1T	71.1	51.93	0.126	5,370,228	5,032
Citrobacter braakii ATCC 51113	67.7	51.90	0.143	5,575,578	5,293
Citrobacter portucalensis A60	64.3	52.01	0.130	4,916,306	4,559
Citrobacter freundii NBRC 12681	64.1	51.66	0.168	4,902,704	4,604
Citrobacter meridianamericanus BR102	61.7	51.31	0.211	5,375,905	5,011
Citrobacter youngae CCUG 30791	60.5	51.79	0.169	4,814,018	4,556
Citrobacter pasteurii CIP 55.13	60.4	51.80	0.158	4,986.623	4,774
Kluyvera cryocrescens NBRC 102467	35.8	53.85	0.132	5,044,663	4,702
Enterobacter pseudoroggenkampii 155092	29.7	55.61	0.081	4,860,215	4,591
Enterobacter dykesii E1T	29.6	55.85	0.077	4,509,323	4,161
Enterobacter pasteurii A-8	28.2	56.41	0.082	4,810,455	4,376
Silvania confinis H4N4	27.8	55.68	0.079	4,864,404	4,524
Silvania hatchlandensis H19S6	27.7	55.90	0.078	4,773,628	4,393
Leclercia tamurae H6S3	27.5	56.37	0.086	4,703,391	4,348
Leclercia adecarboxylata NBRC 102595	27.4	55.57	0.082	4,991,157	4,630
Enterobacter kobei ATCC BAA-260	23.3	55.46	0.153	4,699,704	4,424
Buttiauxella brennerae ATCC 51605	18.0	50.63	0.154	4,758,272	4,311

Antibiotic resistance genes were identified in *C. werkmanni* SAS genome using the Comprehensive Antibiotic Resistance Database (CARD). Results showed that the genome contained five major genes involved in the resistance to several antibiotic classes which included fluoroquinolone, cephamycin, cephalosporin, glycopeptides, polypeptide antibiotics, and tetracyclines as shown in Table 4. Figure 4 describes a comparative genomic analysis of *C. werkmanii* SAS with respect to eleven closely related bacterial species. The blue circle at the center of the diagram represents the reference genome of *C. werkmanii* SAS around which the comparison was made. Surrounding the blue circle are rings that display two key features of the genome. The GC content and the GC skew that measures the asymmetry in the distribution of guanine and cytosine bases in the genome.

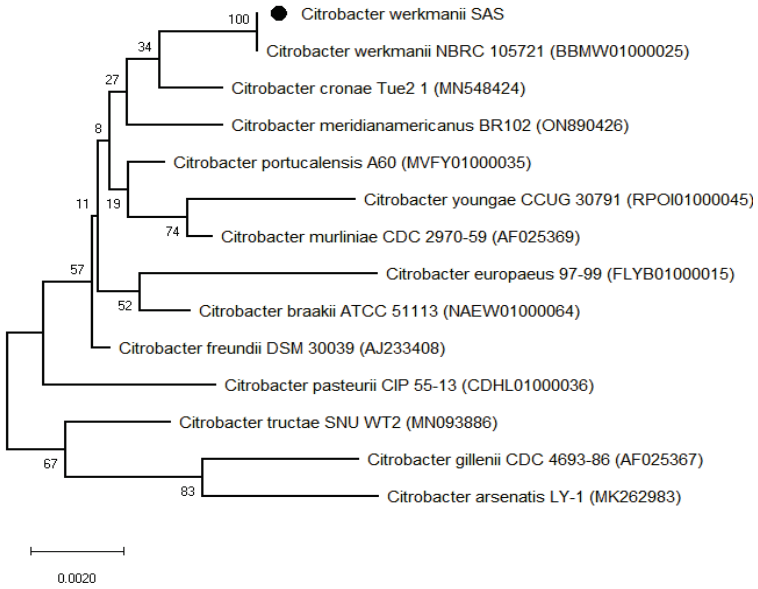

Fig. 3. Phylogenetic trees constructed using the neighbor-joining method illustrate the relationships among *Citrobacter werkmanii* SAS (marked with a black circle) and its closely related strains, based on 16S rRNA sequences analyzed with MEGA-11 software, featuring a scale length of 0.002; the proportion of replicate trees where the related strains grouped together in the bootstrap analysis (100 replicates) is indicated next to the branches

Table 3
Most closely related bacterial species with their accession numbers that show homology with *Citrobacter werkmanii* SAS collected from NCBI database based on 16S rRNA

Species name	Strain name	Accession No.	Similarity, %
Citrobacter werkmanii	NBRC 105721	BBMW01000025	100
Citrobacter portucalensis	A60	MVFY01000035	99.73
Citrobacter cronae	Tue2_1	MN548424	99.66
Citrobacter freundii	DSM 30039	AJ233408	99.52
Citrobacter murliniae	CDC 2970-59	AF025369	99.52
Citrobacter meridianamericanus	BR102	ON890426	99.52
Citrobacter braakii	ATCC 51113	NAEW01000064	99.32
Citrobacter tructae	SNU WT2	MN093886	99.32
Citrobacter youngae	CCUG 30791	RPOI01000045	99.25
Citrobacter pasteurii	CIP 55.13	CDHL01000036	99.1
Citrobacter europaeus	97-99	FLYB01000015	99.1
Citrobacter gillenii	CDC 4693-86	AF025367	98.7
Citrobacter arsenatis	LY-1	MK262983	98.5

Table 4
Antibiotic resistance determinants identified in the genome of *Citrobacter werkmanii* SAS utilizing the CARD database

Antibiotic resistance determinant	Antibiotic group	Mechanism of antibiotic resistance	Expected phenotype	Identity, %
qnrB12	fluoroquinolone antibiotic	antibiotic target protection	ciprofloxacin, levofloxacin, moxifloxacin, gatifloxacin, nalidixic acid, norfloxacin, sparfloxacin	100.00
CMY-159	cephamycin	antibiotic inactivation	unknown	98.43
acrB	fluoroquinolone antibiotic, cephalosporin, glycylcycline, penam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	antibiotic efflux	tigecycline, acriflavine, tetracycline, rifampin, chloramphenicol, ampicillin, cefalotin, triclosan	94.57
pmrF	peptide antibiotic	antibiotic target alteration	polymyxin B	88.16
vanG	glycopeptide antibiotic	antibiotic target alteration	vancomycin	36.84

The next rings represent the genomes of other closely related species indicated in different colors. Areas where the rings do not have color indicate that a particular genomic region is absent in the genome of that species. The absence of certain genomic regions in one or more species could be significant for understanding genetic variations, adaptations, or evolutionary processes.

Discussion

The genome of *C. werkmanii* SAS exhibits unique features consistent with its potential as a pathogenic or environmental bacterium. The draft genome sequence comprises 5,072,546 base pairs (bp) and exhibits a GC content of 51.99%. The parameters indicative of genomic stability and adaptability are distributed across 61 contigs, with the largest contig measuring 941,705 bp and the smallest measuring 528 bp. The N_{50} value of 351,029 indicates the assembly's robustness, emphasizing the significant contribution of large contigs to the genome's completeness. These results come in agreement with Zhou et al. (2022) and Zhou (2017) who found that the genome size of *C. freundii* CD-9 and *C. werkmanii* BF-6 were 5.33 and 4.92 Mb, respectively. This shows that the sequence of our strain was completely covered.

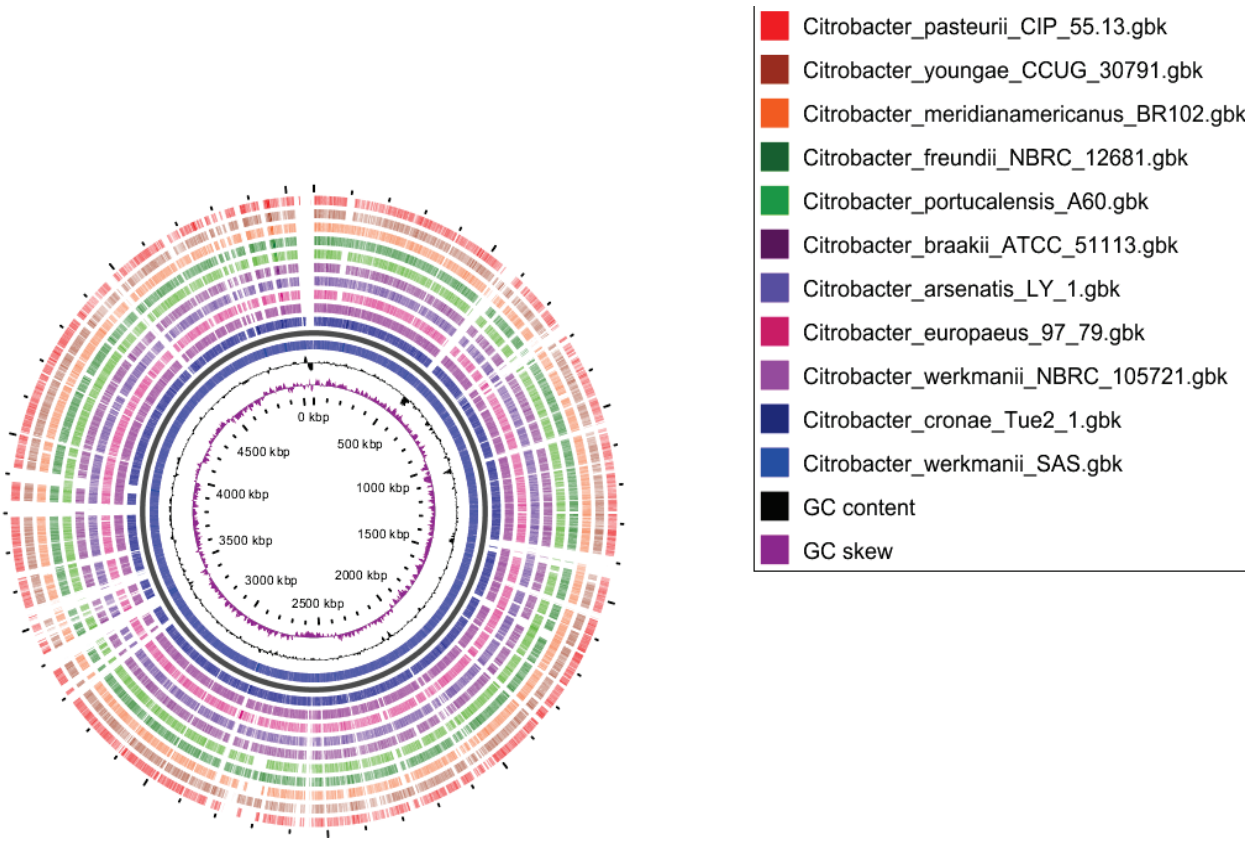

Fig. 4. The genome of *Citrobacter werkmanii* SAS compared with genomes of eleven closely related bacterial species: the innermost blue circle denotes the genome of *C. werkmanii* SAS; the rings illustrate GC content (black) and GC skew (purple); the subsequent rings illustrate the genomes of other closely related species, each represented in distinct colors; areas lacking color in the ring signify the nonexistence of the region and highlight the variations among the genome sequences

Using the RAST server we were able to identify 4,793 coding sequences and 71 RNA genes. The genes belonged to multiple subsystems, making substantial contributions to fundamental metabolic pathways. In the genome of *C. werkmanii* SAS, a total of 403 genes for carbohydrate metabolism were observed, which indicates the versatility of this strain in using different carbon sources. Protein metabolism includes 247 genes, which suggests that protein metabolism is also efficient in terms of protein synthesis and degradation. Moreover, it is evident that 184 genes were involved in producing cofactors, vitamins, prosthetic groups, and pigments necessary for the maintenance of redox balance, coenzyme synthesis, and possible interactions with host systems or environmental niches. These results are in accordance with Rafiqul (2012), who noted that the higher number of genes was connected with carbohydrate metabolism (835), amino acid derivatives (468), coenzymes, vitamins, prostheses, and pigment biosynthesis (324). The above characteristics illustrate the metabolic plasticity and adaptability of *Citrobacter werkmanii* SAS, which might be of ecologically or pathogenic importance.

The importance of detecting antibiotic resistance genes (ARGs) in bacterial genomes is to understand the process that enables microbes to resist antibiotics. CARD is a new technique used for discovering and studying antibiotic resistance genes, providing insights into proven resistance mechanisms and their genetic components. CARD technology uses a specific selected set of thoroughly tested resistance genes and processes to match microbial genome sequences with these known resistance determinants (Papp & Solymosi, 2022). Our results highlights the multidrug-resistant capabilities of *C. werkmanii* SAS, particularly in its resistance to fluoroquinolones and cephalosporins. The extensive efflux capabilities of the acrB gene highlight its significant role in the multidrug resistance capability of this strain. The low identity of vanG necessitates additional experiments to ascertain its functional implications. If these studies prove the role of vanG, this will lead to the discovery of a novel protein involved in resistance. No plasmids were detected in the genome of *C. werkmanii* SAS, however, many studies have shown acquisition of plasmids in *C. werkmanni* which necessitates further genetic studies on this strain to identify the location of antibiotic resistance genes whether on the chromosome or the plasmid (An et al., 2016; Campana et al., 2022).

It appears that the SAS genome contains an equal quantity of genes in leading and lagging strands and showed occasional clustering (likely operons) of genes in the two strands. A consistent GC content was observed in the SAS genome, although low GC content genomic regions were found at several locations. Those regions are complementary to the regions without color in the surrounding rings (compared genomes that indicate the absence of the region and the difference among the genome sequences), which is indicative of a possible horizontal gene transfer or insertions in those genomic locations.

The TYGS utilizes the most reliable methods and cutting-edge estimates in the genomic era to identify the closest type bacterial genome sequences that have validly published names (Saeed et al., 2024). The findings from isDDH indicate that the isDDH value of *C. werkmanii* SAS is ≥70%, surpassing the threshold when compared to the type strain *C. cronae* Tue2-1T and *C. werkmanii* NBRC 105721; this suggests a strong genetic relationship among the three genome sequences. The similarity of isDDH to *C. cronae* Tue2-1T indicates a potential close evolutionary relationship or possible taxonomic ambiguities between *C. cronae* and *C. werkmanii*, suggesting the need for further investigation.

Conclusion

This study illustrates that utilizing whole genome sequencing on specific clinical isolates serves as an effective method for uncovering the fundamental resistance mechanisms and evolutionary processes occurring within individual patients in a healthcare environment. Five antibiotic resistance genes resistant to severalclasses of antibiotics were identified using the CARD server. The classes included fluoroquinolone, cephamycin, cephalosporin, glycopeptides, polypeptide antibiotics, and tetracyclines. A consistent GC content was observed

in the SAS genome; however, several regions with low GC content were identified at various locations. The regions serve as indicators of potential horizontal gene transfer or insertions at specific genomic locations, offering epidemiological evidence that horizontal gene transfer between various species has taken place in hospital environments. The identity of the antibiotic resistance gene, vanG, was found to be low when compared to other published vanG genes, indicating the need for further experiments to determine its role. If these studies demonstrate the role of vanG, this will result in the identification of a new protein associated with resistance.

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