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Research article

First identification and genomic features of multidrug-resistant *Citrobacter freundii* ST669 strain isolated from a domesticated duck in Bangladesh

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ABSTRACT

Objectives: Citrobacter freundii is a prevalent source of nosocomial infections and a well-known cause of diarrheal diseases. In recent years, it has also become increasingly resistant to various antimicrobials. In this study, we screened and characterized a multidrug-resistant (MDR) *C. freundii* isolate obtained from a domesticated diseased duck to better understand the genetic features, molecular epidemiology, and underlying factors linked to the antimicrobial resistance genes (ARGs) and virulence factor genes (VFGs) of the isolate.

Methods: The *C. freundii* BAU_TM8 strain was isolated using culturing, staining, biochemical, polymerase chain reaction, and Matrix-assisted laser desorption/ionization-time of flight methods. The MDR properties of the strain were determined by a disk diffusion test. The genomic sequence of *C. freundii* BAU_TM8 was performed using the Illumina NextSeq2000 platform. The ARGs, VFGs, and genomic functional characteristics of the *C. freundii* BAU_TM8 strain were identified using several open-source databases.

Results: The sequence type of this strain was ST669, and the pathogenicity index of the strain was 0.919. Moreover, the strain had an estimated genome length of 5,797,806 bp, harboring 62 contigs, a G + C content of 54.32 %, and five contig L50s with an N50 value of 443,947 bp. Using phylogenetic analysis, this strain was closely related to two strains isolated from human and environmental samples in the USA and China despite huge geographical distances. The *C. freundii* BAU_TM8 strain consisted of 40 AGRs encoding resistance to 19 antimicrobial categories, e.g., fluoroquinolones, macrolides, folate pathway antagonists, aminoglycosides, tetracyclines, cephalosporins, and others. According to the phenotypic assay and genome sequence, the sensitivity and specificity of resistance profiles of the strain were 100 % and 20 %, respectively. Moreover, the virulence factor database detected 66 VFGs in this strain. This strain contained 1581

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subsystems, having 33 % subsystem coverage and 2275 genes encoding amino acid derivatives, carbohydrate metabolism, protein metabolism, cofactors, vitamins, prosthetic groups, pigments, respiration, motility and chemotaxis, stress response, DNA metabolism, nucleosides and nucleotides, and others.

Conclusions: To the best of our knowledge, this is the first WGS report of *C. freundii* from a domesticated duck in Bangladesh. The ubiquitous occurrence of ARGs and VFGs in the *C. freundii* BAU_TM8 strain detected in this study highlights the growing concern about antimicrobial resistance in humans, animals, and environments.

1. Introduction

Citrobacter freundii, belonging to the family *Enterobacteriaceae*, is a rod-shaped Gram-negative *Bacillus* that is facultatively anaerobic. Although the species is infrequently isolated, it is often found in environments (e.g., water, soil, etc.) and in the intestines of humans and animals, where it is increasingly being implicated in a wide range of both superficial and serious, potentially fatal, opportunistic infections [1]. *Citrobacter* isolates are not just low-virulence environmental contaminants; they are also a leading cause of diseases affecting the urinary system, liver, biliary tract, peritoneum, colon, bone, lungs, heart, soft tissues, and meninges [2]. *Citrobacter* septicemia has a mortality rate ranging from 33% to 48 % [3]. They can spread infections linked to healthcare, particularly in children and people with compromised immune systems [4]. Those infants who manage to endure the ordeal may suffer from severe brain damage that leaves them severely mentally retarded, epileptic, or partially paralyzed [5]. *C. freundii* isolates are frequently transmitted via the faecal-oral route. Eggs, raw or undercooked meat, and slaughterhouse carcasses are all potential sources for the zoonotic bacterium *C. freundii*, which can be spread to humans from poultry sources [6].

Antimicrobial resistance (AMR) has become a growing public health crisis worldwide [7]. The widespread and often careless use of antibiotics has led to the emergence of multidrug-resistant (MDR) bacteria in a variety of settings [8]. There has been a global rise in the occurrence of antibiotic-resistant *C. freundii*, and certain strains have been found to possess extended-spectrum beta-lactamase (ESBL) and plasmid-mediated quinolone resistance (PMQR) genes [9]. Infants, people with impaired immune systems, and aged people are disproportionately prone to contracting *C. freundii* strains resistant to multiple antimicrobials [10]. Multiple virulence factors are present in *C. freundii*, and they are thought to be ways in which bacterial pathogens exhibit their pathogenicity and aid in their persistence in hosts [11]. Toxins, including Shiga-like toxins, heat-stable toxins, and a cholera toxin B subunit homolog, are the primary virulence determinants in *C. freundii* [12]. Proteolysis, hemolysis, and biofilm production are some of the additional *Citrobacter*-related virulence factors [13].

The poultry sector in Bangladesh, especially chickens and ducks, has developed into a specialized subset of the country's overall animal production industry by offering low-cost and conveniently accessible wholesome protein in the form of meat and eggs [14,15]. The favorable climate, natural feed resources, and swimming water availability in Bangladesh make it an ideal environment for duck farming [16]. In Bangladesh, poultry farmers are in direct contact with domesticated ducks. As a result, the presence of any pathogens in ducks poses a public health threat by being transferred to humans via direct contact or the food chain. *C. freundii* is not routinely isolated and examined in Bangladesh because of its relatively low prevalence and lack of comprehensive research on antimicrobial resistance. To address this issue, in this study, we analyzed an MDR *C. freundii* BAU_TM8 strain isolated from a domesticated duck in Bangladesh using its whole genome sequencing. We also evaluated its genetic characteristics, antimicrobial resistance, and virulence profiles.

2. Methods

Ethics approval

The research was conducted in accordance with the "ARRIVE" guidelines, and all applicable regulations and guidelines were followed during the study. Animal studies were conducted in strict accordance with all the guidelines and regulations of the "Animal Welfare and Experimentation Ethics Committee" of Bangladesh Agricultural University, Mymensingh 2202, Bangladesh. The university's ethics committee (Animal Welfare and Experimentation Ethics Committee, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh) approved all the methodologies and related protocols used in this study. The protocols were registered under the following approval number: AWEEC/BAU/2020(10). The authors affirm that all the techniques employed aligned with relevant regulations and guidelines for the research article.

2.1. Isolate retrieval

The *Citrobacter freundii* BAU_TM8 strain was retrieved from a cloacal swab sample of a domesticated diseased duck (having several symptoms, such as whitish or greenish diarrhea, leg weakness, and headshaking) in Bangladesh in March 2020. The sample was cultured in nutrient broth, inoculated on xylose-lysine deoxycholate (XLD) (HiMedia, India), and incubated overnight at 37 °C, followed by selecting pure colonies. Yellow colonies with a black center on XLD were considered presumptive *Citrobacter* spp. These colonies were then screened to check their morphological characteristics using Gram's staining and biochemical tests for further

confirmation [17]. The identification of *Citrobacter* spp. was confirmed by a polymerase chain reaction (PCR) assay targeting a specific 16S rRNA gene [18]. Finally, a matrix-assisted laser desorption/ionization-time of flight mass spectrometry assay [19] was employed to detect *C. freundii* BAU_TM8.

2.2. Antibiotic susceptibility test

The *C. freundii* BAU_TM8 strain was screened for antibiotic susceptibility on Mueller Hinton agar (HiMedia, India) to determine its antibiogram profiles using the disk diffusion method [20]. The McFarland 0.5 concentration was maintained during the cultural growth of the isolate. Then, the isolate was tested against 13 antibiotic classes (20 antibiotics): ansamycins (rifampin), folate pathway antagonists (cotrimoxazole), fluoroquinolones (ciprofloxacin and levofloxacin), aminoglycosides (gentamicin and streptomycin), tetracyclines (tetracycline), macrolides (azithromycin and erythromycin), cephalosporins (ceftriaxone, cephalexin, cefotaxime, and ceftazidime), penicillins (ampicillin), amphenicols (chloramphenicol), phosphonic acid (fosfomycin), nitrofurans (nitrofurantoin), monobactam (aztreonam), and carbapenems (imipenem and meropenem). The reference strain *Escherichia coli* ATCC 25922 was used to perform quality control. The Clinical and Laboratory Standard Institute (CLSI) guidelines were employed to interpret the outcomes (sensitive, intermediate, and resistant) [21]. The isolate showing three or more classes of antibiotics was considered MDR [22].

2.3. DNA extraction and whole genome sequencing for C. freundii BAU_TM8 strain

The genomic DNA from the *C. freundii* BAU_TM8 strain was extracted using the Qiagen DNA mini kit (QIAGEN, Hilden, Germany). NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Fisher, Waltham, MA, USA) was utilized to assess DNA concentration and purity. The extracted genomic DNA was then sent to the Child Health Research Foundation (Dhaka, Bangladesh) for genome sequencing (WGS). The Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA) was used to generate the DNA libraries, and all steps were carried out according to the manufacturer's instructions. Finally, the sequencing was performed on the Illumina NextSeq2000 platform (read length: 2×150 bp).

2.4. Genome assembly, annotation, and data analysis for C. freundii BAU_TM8 strain

Genome assembly was done using Unicycler v0.4.9 [23] and checked using Bandage v0.8.1 [24]. This is followed by a preliminary step of trimming the raw paired-end reads with Trimmomatic.v0.39 [25] (leading: 20, sliding window: 4:20:20, trailing: 20, and minlen = 36) to remove Illumina adapters and phiX reads from the data set. Quality assessment was performed using FastQC.v0.11.7 [26]. Genome annotation was done using Prokka v1.14.6 [27] and the Pathosystems Resource Integration Center (PATRIC) [28]. The SpeciesFinder 2.0 [29], MLST 2.0 [30], and PathogenFinder 1.1 [31] opensource tools were used to identify the species (i.e., *C. freundii*), sequencing typing of the isolate (i.e., *C. freundii* BAU_TM8) and pathogenicity of the isolates (i.e., *C. freundii* BAU_TM8), respectively. Moreover, the *C. freundii* BAU_TM8 genome was compared with NCBI-referenced another whole genome sequence, namely *C. freundii* MRSN 12115 (unpublished) (NCBI sequence accession number JYFZ02000001, BioProject: PRJNA273931). Proksee [32] was used to visualize the circular annotation of our strain as well as the comparison between our strain and *C. freundii* MRSN 12115 genomes.

2.5. Genome functional potentials analysis of C. freundii BAU_TM8 strain

Antibiotic resistance genes (ARGs) prediction, virulence factor genes (VFGs) profiling, and metabolic function analysis were all performed using the annotated WGS data of the *C. freundii* BAU_TM8 strain. The RAST (Rapid Annotation Using Subsystem Technology) server provided data on the distribution of genes in various categories (genomic functional features). The Resistance Gene Identifier (RGI) from the Comprehensive Antibiotic Resistance Database (CARD) [33] and the virulence factor database (VFDB) [34] open-access databases were utilized to detect ARGs and VFGs, respectively. The ARGs selection criteria were set to perfect (100 % identity) and strict (>95 % identity) hits only to the curated reference sequences in the CARD databases. The draft genome of the *C. freundii* BAU_TM8 strain was also annotated using the RAST server [35]. Moreover, the visualization of different ARGs present in our strain was performed using Proksee [32] and the percentage of virulence genes in each virulence factor category was visualized in Excel 365 (Microsoft Office 365, Redmond, Washington, USA).

2.6. Sensitivity and specificity of the antibiotic resistance profiles of the C. freundii BAU_TM8 strain

As previously described [36], the concordant between phenotype and genotype was established when the isolate, displaying resistance or susceptibility to a drug in the disk diffusion test, also possessed or lacked associated ARGs for that drug, respectively. True positives (TP) occurred when resistance was phenotypically observed and corresponding ARGs were present, while true negatives (TN) were noted when susceptibility was observed and no corresponding ARGs were found. TP and TN outcomes signified that in-silico resistance predictions based on WGS aligned with actual phenotypic outcomes. False negatives (FN) referred to isolates with phenotypic resistance but lacking relevant resistance-conferring ARGs, whereas false positives (FP) denoted isolates with phenotypic susceptibility yet harboring ARGs linked to the drug. FP and FN results indicated a mismatch between in-silico predictions and observed phenotypic resistance. The sensitivity and specificity of the *C. freundii* BAU_TM8 strain were calculated as follows:

$$\text{Sensitivity} = \frac{TP}{TP + FN} \times 100\%$$

$$\text{Specificity} = \frac{TN}{TN + FP} \times 100\%$$

2.7. WGS phylogenetic relatedness of worldwide strains with the C. freundii BAU_TM8 strain

The *C. freundii* BAU_TM8 strain was compared with a total of 41 representative strains (Supplementary Table S1) from different countries of the world, including Bangladesh (n = 2), the USA (n = 12), China (n = 13), South Africa (n = 3), Switzerland (n = 2), Japan (n = 1), Denmark (n = 1), Australia (n = 1), Malaysia (n = 2), Brazil (n = 1), Spain (n = 1), Sri Lanka (n = 1), and Sweden (n = 1). The relatedness was shown using phylogenetic analysis. The phylogenetic relationships among the user-uploaded multiple genome sequences were determined using a bacterial whole-genome sequence typing and source tracking tool called BacWGSTdb.v.2.0 [37]. Here, we combined the results of several genome analyses into a single model to better predict the presence of genes associated with virulence and resistance to antimicrobials. The phylogenetic tree was constructed using the approximate maximum likelihood technique in iTOL.v.5 [38] from the various genomic sequence alignments. The phylogenetic tree was updated to include the annotated ARGs predicted by CARD analysis [33] from the genome sequences of global strains. Finally, the heatmap was prepared using PowerPoint 365 (Microsoft Office 365, Redmond, Washington, USA) to perfectly and strictly hit antibiotic resistance genes under different antimicrobial categories.

3. Results

3.1. Genome characteristics of the C. freundii BAU_TM8 strain

The isolate was detected as *Citrobacter freundii* BAU_TM8 by SpeciesFinder 2.0. PathogenFinder 1.1 revealed that the probable pathogenicity index of the isolate was 91.9 %, indicating that the strain might show a higher pathogenic characteristic. The sequence type of the genome was ST669 using the MLST server. The assembly revealed that the *Citrobacter freundii* BAU_TM8 genome had 62

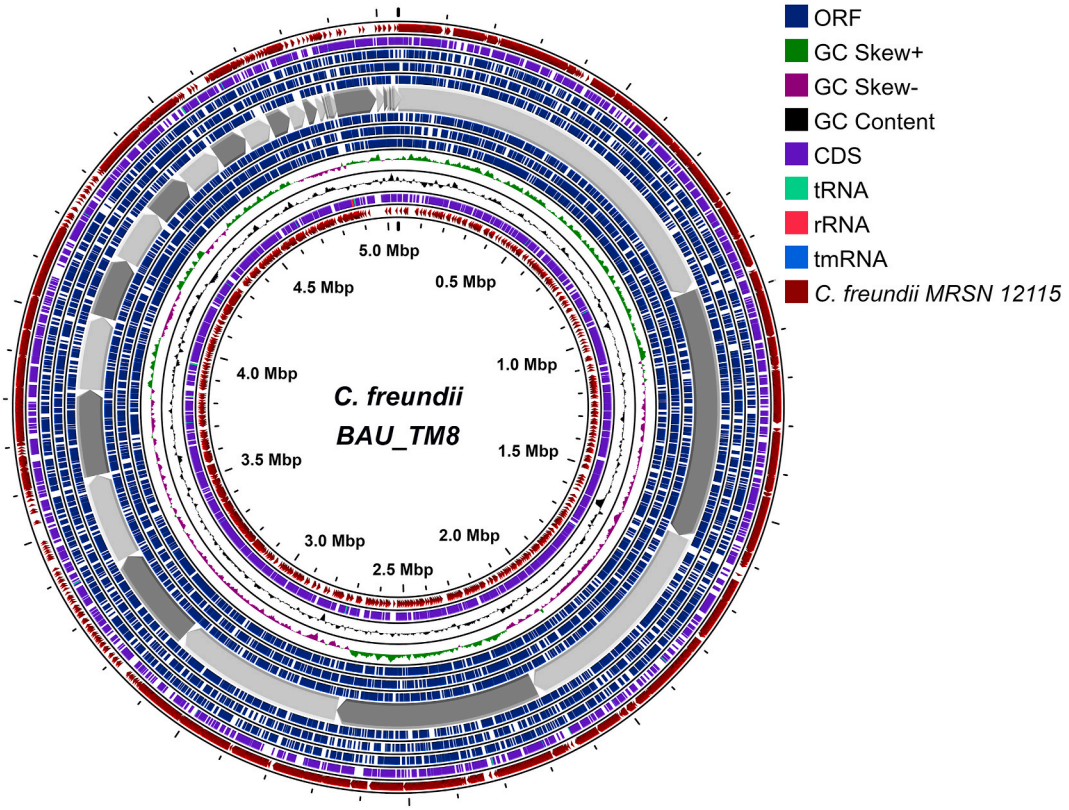


Fig. 1. Genome circularization of *C. freundii* BAU_TM8 and comparison to *C. freundii* MRSN 12115 (JYFZ02000001). Here, Mbp = mega base pairs, CDS = coding sequences, ORF = open reading frames, GC = guanine-cytosine, tRNA = transfer RNA, rRNA = ribosomal RNA, tmRNA = transfer messenger RNA.

contigs, an estimated genome length of 5,797,806 bp, and an average guanine-cytosine content of 54.32 %. The N50 length of the genome, which is defined as the shortest sequence length at 50 % of the genome, is 443,947 bp. The L50 count, which is defined as the smallest number of contigs whose length sum produces N50, is 5. Fig. 1 displays a physical genome map of *C. freundii* BAU_TM8 in relation to the reference genome *C. freundii* MRSN 12115, indicating a similarity of almost 100 %.

A circular genome map (Fig. 2) was generated by PROKKA using the PATRIC. This map revealed several characteristics of the genes. For example, this genome had 5767 protein-coding sequences (CDS), 85 transfer RNA (tRNA) genes, and 6 ribosomal RNA (rRNA) genes. The genome also contained 4717 and 1050 functional and hypothetical proteins, respectively. The proteins with functional assignments comprised 1495 with Enzyme Commission numbers, 1253 with Gene Ontology assignments, and 1083 with KEGG pathway mapping. Moreover, this genome has 4780 genus-specific and 5502 cross-genus protein families.

3.2. Antimicrobial resistance profiles in the C. freundii BAU_TM8 strain

The *C. freundii* BAU_TM8 strain was determined as MDR in nature, showing phenotypic resistance to 11 out of 13 antimicrobial categories and 15 out of 20 antimicrobial agents, including rifampin, cotrimoxazole, erythromycin, azithromycin, ciprofloxacin, levofloxacin, gentamicin, ampicillin, tetracycline, aztreonam, cephalexin, ceftriaxone, cefotaxime, ceftazidime, and imipenem (Table 1).

Annotation of ARGs in the *C. freundii* BAU_TM8 genome with the CARD system was performed to investigate the potential genetic reasons underlying the multidrug resistance of the strain. In the *C. freundii* BAU_TM8 genome, the efflux pump that confers resistance to various antibiotics was detected to be the predominant resistance class. The bacterial genome also contains several putative MDR transporter genes belonging to the ATP-binding cassette (ABC) family, the resistance nodulation division (RND), and the major facilitator superfamily (MFS) (Supplementary Table S2). The resistomes of *C. freundii* BAU_TM8 consisted of 40 AGRs under encoding resistance to 19 antimicrobial categories, such as carbapenems (*marA* and *soxS*), phosphonic acid (*mdtG*, *GlpT*, and *UhpT*), tetracyclines (*tetA*), folate pathway antagonists (*dfrA27*, *sulI*), macrolides (*mphA*), fluoroquinolones (*qnrS1*, *gyrA*), ansamycins (*arr-3*), aminoglycosides (*aph(3″)-Ib*, *aac(3)-IId*, *aph(6)-Id*, *aadA16*, *aac(6′)-Ib-cr6*), cephalosporins ($bla_{\text{TEM-1}}$), cephamycins ($bla_{\text{CMY-51}}$), and others (Table 1). In addition, the mutant penicillin-binding protein (*PBP3*) gene harboring mutations encoding beta-lactam antibiotic resistance was found (Table 1).

In this study, resistance genes identified from WGS correlated with phenotypic testing results with an overall sensitivity and specificity of 100 % and 20 %, respectively. However, we found discrepancies for fosfomycin, chloramphenicol, and meropenem. The strain was phenotypically sensitive to these antimicrobial agents but showed genotypic resistance to them using the WGS analysis (Table 1).

3.3. Virulence profiles in the C. freundii BAU_TM8 strain

The VFDB database detected about 66 VFGs in the *C. freundii* BAU_TM8 genome (Supplementary Table S3). The genes linked with fimbrial adherence determinants contained the highest number of genes (28 genes), followed by adherence (11 genes), iron uptake (10

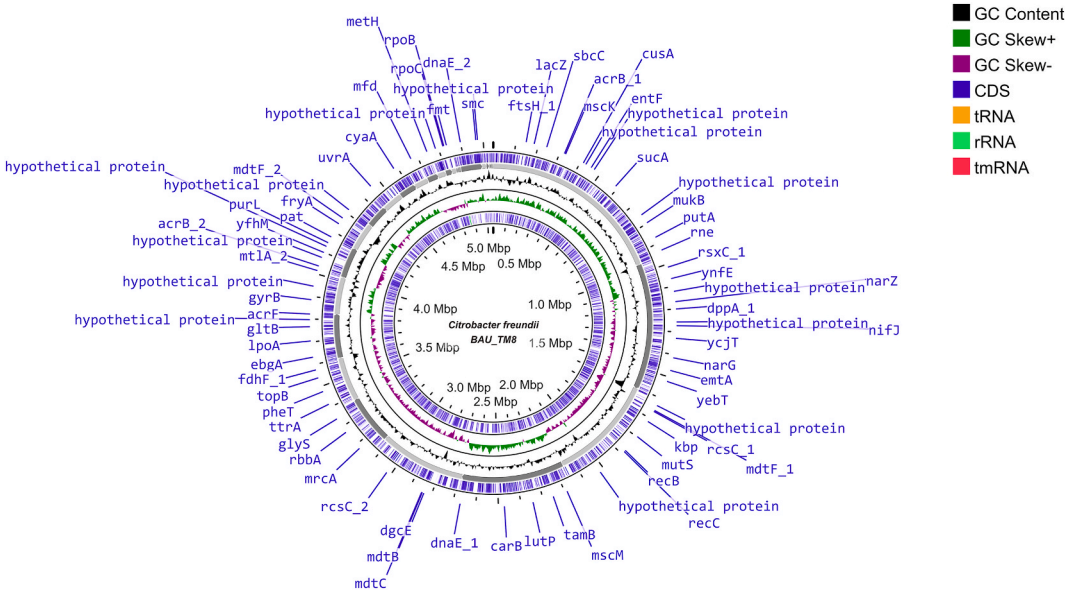


Fig. 2. A circular diagram of the genome of the *Citrobacter freundii* strain BAU_TM8. Genes encoding core and putative proteins are annotated with Prokka on both strands, which are displayed in the outermost ring (blue).

Table 1
The AMR profiles and their corresponding ARGs predicted by disk diffusion method and CARD in *Citrobacter freundii* BAU_TM8 strain.

Antimicrobial categories	Name of antimicrobial agents	Outcomes of DDT (R/I/S)	Predicted AGRs using CARD analysis
Ansamycins	Rifampin	R	*arr-3*, *acrA*, *acrB*, *KpnE*, *KpnF*, *marA*, *AcrAB-TolC*, *soxS*
Folate pathway antagonists	Cotrimoxazole	R	*dfrA27*, *rsmA sulI*
Macrolides	Erythromycin	R	*mphA*, *KpnE*, *KpnF*, *H-NS*, *CRP*
	Azithromycin	R	
Fluoroquinolones	Ciprofloxacin	R	*qnrS1*, *acrA*, *acrB*, *marA*, *H-NS*, *emrB*, *emrR*, *rsmA*, *CRP*, *aac(6′)-Ib-cr6*, *parC*, *gyrA*, *AcrAB-TolC*, *soxS*
	Levofloxacin	R	
Aminoglycosides	Gentamicin	R	*aac(3)-IId*, *kdpE*, *KpnE*, *KpnF*, *baeR*, *aph(6)-Id*, *aph(3″)-Ib*, *aac(6′)-Ib-cr6*, aadA16
	Streptomycin	I	
Penicillins	Ampicillin	R	bla_{TEM-1}, *acrA*, *acrB*, *marA*, *H-NS*, *CRP*, *PBP3*, *AcrAB-TolC*, *soxS*
Tetracyclines	Tetracycline	R	*tet(A)*, *mdfA*, *acrA*, *acrB*, *KpnE*, *KpnF*, *marA*, *H-NS*, *AcrAB-TolC*, *soxS*
Phosphonic acid	Fosfomycin	S	*mdtG*, *GlpT*, *UhpT*
Monobactams	Aztreonam	R	bla_{TEM-1}, *marA*, *soxS*
Cephalosporins	Cephalexin	R	bla_{TEM-1}, *acrA*, *acrB*, *KpnE*, *KpnF*, *marA*, *H-NS*, *PBP3*, *AcrAB-TolC*, *soxS*
	Ceftriaxone	R	
	Cefotaxime	R	
	Ceftazidime	R	
Amphenicols	Chloramphenicol	S	*acrA*, *acrB*, *marA*, *rsmA*, *AcrAB-TolC*, *soxS*
Nitrofurans	Nitrofurantoin	S	–
Carbapenems	Imipenem	R	*marA*, *soxS*
	Meropenem	S	
Glycopeptides	–	–	*vanG*
Cephamycins	–	–	*marA*, *H-NS*, bla_{CMY-51}, *PBP3*, *soxS*
Polypeptides	–	–	*KpnE*, *KpnF*, *PmrF*
Glycylcyclines	–	–	*acrA*, *acrB*, *marA*, *AcrAB-TolC*, *soxS*
Nitroimidazoles	–	–	*msbA*
Aminocoumarins	–	–	*mdtB*, *mdtC*, *baeR*

Here, R = Resistant, I = Intermediate, S = Sensitive, DDT = Disk diffusion test, CARD = Comprehensive Antibiotic Resistance Database, ARGs = Antibiotic Resistance Genes.

genes), and non-fimbrial adherence determinants (four genes). In addition, two (3.03 %) VFGs were linked to each regulation and acid resistance properties, and one (1.52 %) VFG was associated with each secretion system, toxin, antiphagocytosis, autotransporter, biofilm-formation, immune evasion, and invasion. Moreover, two virulence genes were linked to unknown virulence factors (Fig. 3).

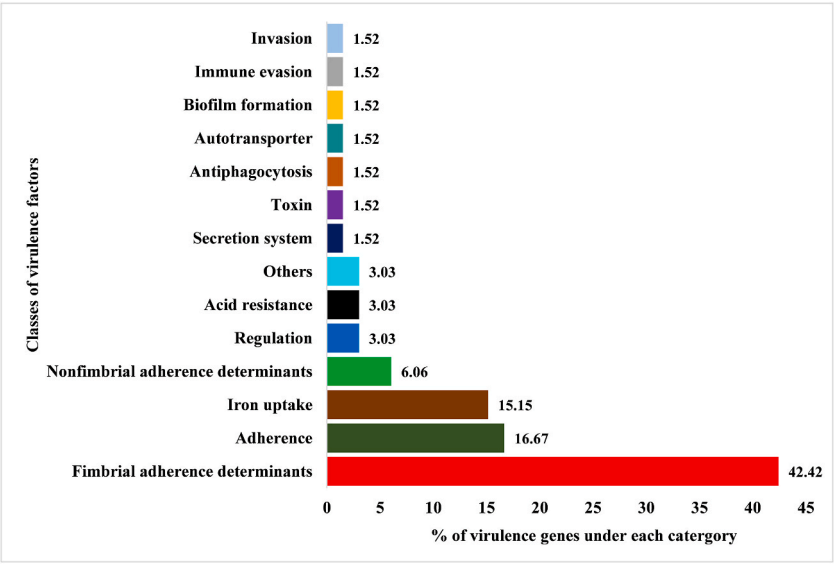


Fig. 3. A summary of the virulence factor genes (VFGs) ascribed to the genome of *C. freundii* BAU_TM8 strain. The VFDB database was utilized to annotate the WGS of the *C. freundii* BAU_TM8 strain. The coverage may be seen, as well as the percentage of each VFG feature in the bar graphs.

3.4. Metabolic functional characteristics in the C. freundii BAU_TM8 strain

The RAST server was also used to search the genome of *C. freundii* BAU_TM8 for functional genes and metabolic pathways. This genome constituted 1581 subsystems with 33 % subsystem coverage and 2275 genes. The highest number of annotated genes were observed in amino acid derivatives (385 genes), followed by carbohydrate metabolism (374 genes), protein metabolism (242 genes), cofactors, vitamins, prosthetic groups, pigments (190 genes), respiration (118 genes), motility and chemotaxis (96 genes), stress response (95 genes), DNA metabolism (83 genes), nucleosides and nucleotides (82 genes), and others. RAST studies also demonstrated the presence of 54 putative virulence, disease, and defense-related genes in the bacterial genome, suggesting its capability to cause fatal human diseases. Among them, 33 genes were related to antibiotic and toxic compound resistance, 14 to invasion and intracellular resistance, five to adhesion, and two to bacteriocins, ribosomally synthesized antibacterial peptides.

3.5. Phylogenetic relatedness of the present strain with other global C. freundii strains

The phylogenetic analysis revealed that the *C. freundii* BAU_TM8 strain shared a close genetic relationship with strains from the USA strain (AMC0703; isolated from human colon content) and China strain (18-1; isolated from river sediment) (Fig. 4). This strain also showed a close phylogenetic relationship with other four isolates, i.e., a Denmark strain (AMA 754, isolated from human pleural fluid), two USA strains (UMB1094 and RU2, isolated from human urinary catheter and human stool samples, respectively), and a China strain (CD-9, isolated from sludge sewage). Fig. 4 further shows that the predominance of ARGs detected in this strain correlates with ARGs found in strains from around the world. The presence of perfectly hit resistant genes harbored in this strain was comparable to that of a Bangladeshi strain isolated from chicken (NR-12), two South African strains isolated from humans (H2730R and ST62:944112508), three China strains isolated from animal and environment (WCHCF65, c196, and Cf.1), two USA strains isolated from human and environment (CFNIH1 and MRSN_11938), a Sweden strain (D8, the source is unknown), and a Denmark strain isolated from human (AMA 754) (Fig. 4 and Supplementary Table S1). Based on the VFDB, the *C. freundii* BAU_TM8 strain (n = 66) had almost similar number of virulence factor genes present in NR-12 (n = 63), L91 (n = 68), A1 (n = 64), GTC_09479 (n = 65), ST62:944112508 (n = 64), FDAARGOS_73 (n = 65), CF1_ST89 (n = 63), AR_0021 (n = 64), and others (Supplementary Table S1).

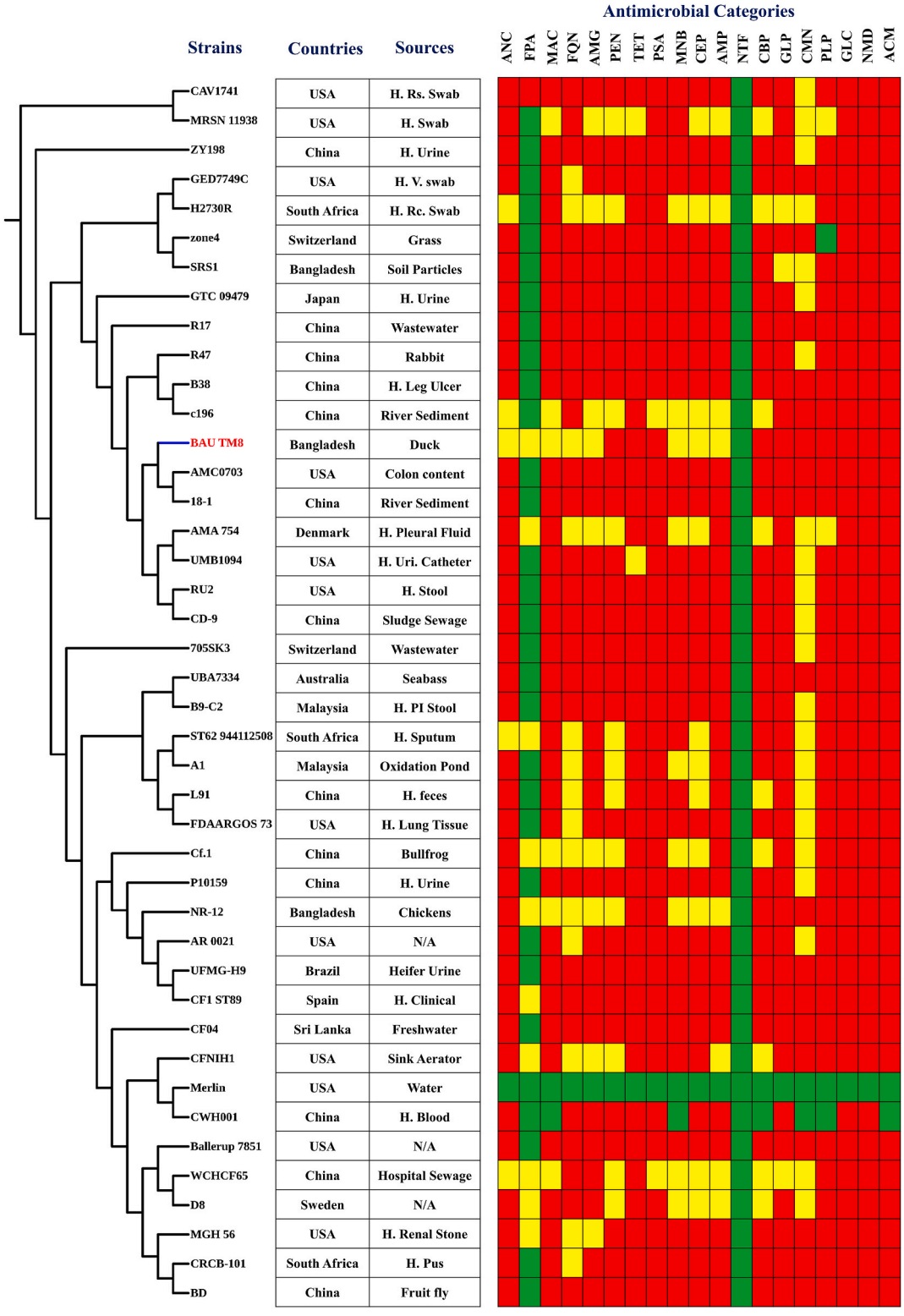
4. Discussion

Citrobacter freundii is a potentially zoonotic and opportunistic pathogen [39] that has been linked to occurrences of gastroenteritis and food poisoning. Antimicrobial-resistant and virulent *C. freundii* can potentially be transferred from poultry to humans via direct contact and the food chain, suggesting a severe public health threat. However, the occurrence of *C. freundii* in poultry, especially in ducks, is still not studied well in Bangladesh. As far as we know, there is no data on the identification and characterization of *C. freundii* in ducks in Bangladesh. Moreover, the harmful consequences of *C. freundii* could be minimized if their evolutionary history, metabolic capability, and VFG and ARG profiles were well understood. Therefore, in the present study, we detected a *C. freundii* BAU_TM8 isolate in a domesticated duck in Bangladesh. We characterized the isolate using WGS to determine its antimicrobial resistance and virulence profiles.

Citrobacter freundii can cause opportunistic infections in people due to its versatile genetic makeup, which allows it to colonize a wide range of habitats [40]. In this study, WGS analysis revealed that the *C. freundii* BAU_TM8 strain had a diverse and dynamic genetic makeup. The genomic characteristics of the *C. freundii* BAU_TM8 genome that have been characterized in the current study are similar to the genome properties of a variety of *C. freundii* strains that have been reported in the past from other countries [41,42]. Moreover, this strain was 100 % similar to a reference genome, *C. freundii* MRSN 12115, isolated from a human sample in the USA. The genome sequencing (using WGS) for phylogenetic analysis allows for a more accurate assessment of the evolutionary relationships between sampled species. Epidemiological surveillance should be automated and strengthened by using this type of analysis.

Citrobacter freundii has developed a higher resistance level to various antimicrobial classes over time [43]. Moreover, AMR and/or ARGs have been exacerbated by the widespread use of multiple antimicrobials for poultry disease control, particularly in lower-middle-income countries, including Bangladesh [44]. In this study, the *C. freundii* BAU_TM8 strain exhibited high antimicrobial resistance in both phenotypic and WGS assays. The genome of *C. freundii* BAU_TM8 appears to contain genes for MDR efflux systems with similar specificities. Among these are the putative MFS and RND family gene sets (*emrB* and *emrR* and *acrA* and *acrB*), which may both transport the cation fluoroquinolone and ansamycin groups of antibiotics, as reported by Yap et al. [1]. However, it is unclear why cells have many coping mechanisms against the same toxic materials. These overlapping specificities of MDR efflux systems are thought to improve bacterial fitness by allowing cells to maximize the removal of target substances [1,45,46]. This also suggests that *C. freundii* BAU_TM8 may have developed widespread resistance to almost all antibiotics used for disease treatment and may have emerged as a pandrug-resistant bacterium in Bangladesh due to the presence of ARGs and similar resistance mechanisms. WGS was also used to learn more about the mechanisms behind AMR, such as the MDR efflux systems, transcriptional activator, probable aminoglycoside efflux pump, intrinsic MDR mechanism, MDR efflux pump outer membrane protein, and MDR proteins. This agreement aligns with previous research and lends credence to the data already available on WGS's reliability in predicting AMR phenotypes [1,6,47,48]. Moreover, this strain contained resistance genes under the cephalosporin and cephamycin classes of antimicrobial agents ($bla_{\text{TEM-1}}$ and $bla_{\text{CMY-51}}$). Resistance genes conferring cephalosporin and cephamycin resistance were also detected in the previous studies conducted in Bangladesh, China, Malaysia, and Taiwan [1,6,9,43]. The presence of resistance genes against a high number of antibiotic classes in this strain suggests quick action to curb the overuse of antimicrobials for treating poultry diseases.

Moreover, phylogenetic analysis between the present strain and selected 41 global strains revealed that this strain was closely



(caption on next page)

Fig. 4. The maximum likelihood core-gene phylogeny of *Citrobacter* species determined by BacWGSTdb is shown at the far left of the picture. The figure also illustrates the sources of the isolates and the countries from which where isolates were come. The heatmap of the figure indicates antibiotic resistance genes under different antimicrobial categories. Yellow color denotes that the isolate harbors perfectly hit resistance genes, the red color indicates that it contains strictly hit resistance genes, and the green color exhibits that the isolate doesn't carry any resistance genes. H. = Human, Rs. = Respiratory, Rc. = Rectal, V. = Vaginal, Uri. = Urine, PI = Preterm infant, N/A = Not applied/found, ANC = Ansamycins, FPA = Folate pathway antagonists, MAC = Macrolides, FQB = Fluoroquinolones, AMG = Aminoglycosides, PEN = Penicillins, TET = Tetracyclines, PSA = Phosphonic acid, MNB = Monobactams, CEP = Cephalosporins, AMP= Amphenicols, NTF = Nitrofurans, CBP = Carbapenems, GLP = Glycopeptides, CMN = Cephamycins, PLP = Polypeptides, GLC = Glycylcyclines, NMD = Nitroimidazoles, ACM = Aminocoumarins.

related to six strains of the USA (n = 3), China (n = 2), and Denmark (n = 1), isolated from human and environmental samples, respectively. Since a few caveats, for example, the phylogeny analysis was conducted on a random assortment of archived *C. freundii* isolates from the NCBI database, we cannot conclude that the ratios of ARGs and phylogeny revealed in the study are significant. However, in this study, we showed the possible utility of genome sequencing to suggest the spread of strains, which might be connected with data from adjacent ARGs and many other details about the strains.

Virulence determinants recognized in *Citrobacter* species were determined in the present strain, including fimbriae, toxin, acid resistance, flagellar motility, iron uptake, antiphagocytosis, etc. Although the virulence genes of the *C. freundii* BAU_TM8 strain show a high degree of conservation, the strain also demonstrates several distinctive characteristics, such as the ability to form biofilms, that may affect its ecological adaptations [1]. In the current study, a total of 66 VFGs were detected in this strain using the VFDB database. We found that this strain is particularly abundant in virulent-strain-specific proteins, as evidenced by the existence of distinct genes encoding for flagellar hook protein (*flg* genes) and flagellar filament structural or regulatory protein (*fli* genes) [1]. These VFGs play an essential role in the development of the pathogenesis of a wide variety of infectious consequences brought on by a variety of Enterobacterales pathotypes, including *Citrobacter* species [9]. This strain harbored several fimbrial adherence determinants and dispensary virulence genes, such as *fimA*, *fimC*, *fimF*, *fimH*, *fimD*, etc., which have a significant role in the invasion of human epithelial cells in the gastrointestinal tract, the urinary tract, and the lungs. In fact, the suppression of the *fimD* gene in *C. freundii* led to a reduction in the organism's ability to adhere to and invade epithelial cells, as demonstrated by an expression analysis [49]. Moreover, it is possible that bacteria with these genes have a higher potential for causing disease and adapting to human environments. Moreover, PathogenFinder exhibited that this strain had a high pathogenicity index (0.919), indicating the isolate might be very highly pathogenic for humans. The present study revealed that the *C. freundii* BAU TM8 strain isolated from a duck expresses multiple VFGs, suggesting it may act as a zoonotic pathogen.

5. Conclusions

We describe the genomic features of an MDR *C. freundii* strain harboring multiple significant ARGs, VFGs, and genomic functional possibilities that may shed light on how AMR genes spread from bacteria to animals, the environment, and eventually to humans. To the best of our knowledge, this is the first study of its kind in Bangladesh using WGS to investigate MDR *C. freundii* in duck samples. Additional research employing a greater number of *C. freundii* isolates could contribute to a better understanding of the diversity of *C. freundii* in Bangladesh and the global spread of the strain.

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Data availability statement

Genome assembly reports from the WGS of the *C. freundii* BAU_TM8 strain have been deposited in GenBank under the accession number JAPNXL000000000, BioProject accession number PRJNA903068, and BioSample accession number SAMN31781978. The JAPNXL000000000.1 version of the WGS of the *C. freundii* BAU_TM8 strain is being described in this study.

CRediT authorship contribution statement

Tarana Ahmed: Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Md Saiful Islam:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization, Validation. **Mohammad Nuruzzaman:** Resources, Methodology, Conceptualization. **Mohammad Sadekuzzaman:** Software, Formal analysis, Data curation. **S.M. Lutful Kabir:** Writing – review & editing, Validation, Supervision. **Md Tanvir Rahman:** Writing – review & editing, Validation, Supervision. **Md Shahidur Rahman Khan:** Writing – review & editing, Validation, Supervision, Resources.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing

interests: Tarana Ahmed reports financial support was provided by Bangladesh Agricultural Research Council. Tarana Ahmed reports a relationship with Bangladesh Agricultural Research Council that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36828.

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