Infection and Drug Resistance

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SHORT REPORT

Genetic characterization of a novel sequence type of multidrug-resistant *Citrobacter freundii* strain recovered from wastewater treatment plant

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Abstract: A multidrug-resistant *Citrobacter freundii* strain R17 was isolated from a wastewater treatment plant in China. Whole-genome sequencing of strain R17 revealed a new sequence type (ST412) chromosome (length 5,124,258 bp) and an Inc FII (Yp) group plasmid pCFR17_1 (length 206,820 bp). A total of 13 antibiotic-resistance genes (ARGs) that confer resistance to eight different antibiotic groups were encoded by strain R17 and 12 of them were carried by plasmid pCFR17_1. These data and analysis suggest that the environment-derived *C. freundii* strains may serve as potential sources of ARGs and highlight the need of further surveillance of this bacteria in the future.

Keywords: *Citrobacter freundii*, multidrug resistant, antibiotic-resistance genes, whole-genome sequencing, wastewater treatment plant, insertion elements

Introduction

Citrobacter freundii is a Gram-negative and facultative anaerobic bacterium which belongs to family Enterobacteriaceae. After its first isolation from soil, *C. freundii* has been found in various natural habitats, as well as intestines of animals and humans.[1–4] Some environment-derived *C. freundii* strains have potential use in many fields. For example, *C. freundii* strain JPG1, which was isolated from a gold mining tailing in China, was resistant to heavy metals and capable of removing copper. Thus, it was suggested to have a great potential in the treatment of copper-rich industrial wastewater.[5] *C. freundii* strain IFO 13545 has the ability to produce bioflocculant which can be used in fields of water supply, wastewater treatment, and food production.[6] Although *C. freundii* was previously recognized as a bacterium of low virulence, it has been demonstrated to be the causative agent of a wide spectrum of infections including diarrhea, pneumonia and septicemia.[7,8] Moreover, there has been a growing body of literature that reported the plasmid-mediated multidrug resistance in *C. freundii*, indicating its threat to human health.[9,10]

Wastewater treatment plants (WWTPs) have been regarded as the major source of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs).[11,12] Previous studies have reported the isolation of several *C. freundii* strains from WWTPs.[13,14] In our investigation of the ARB and ARGs in WWTPs, we obtained a multidrug-resistant strain of *C. freundii*. This strain (R17) was isolated from the sludge of a WWTP that treats wastewater of a pharmaceutical industry in Taizhou, China. Sludge samples were collected from this WWTP in April 2017. Samples were first

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cultured overnight in Mueller-Hinton broth without antimicrobial agents, and then the enriched samples were spread on chromID® ESBL agar plates (bioMérieux, Marcy-l'Étoile, France). After incubating at 37°C for 48 hrs, colonies were picked up for identification using both Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and 16S rRNA sequencing technology as described previously.[4] In the present study, we reported the whole-genome sequencing of *C. freundii* strain R17. Further investigations were conducted on the genetic features and ARGs associated with its resistance phenotypes.

Antimicrobial susceptibility testing of strain R17 was performed using VITEK 2 system employing panel AST-GN-16 (bioMérieux) with *Escherichia coli* ATCC 25922 as control. The results were interpreted according to the standards of the Clinical and Laboratory Standards Institute.[15] Minimum inhibitory concentration (MIC) of strain R17 indicated its resistance to various types of antibiotics, including ampicillin (MIC ≥32 μg/mL), amoxicillin (MIC ≥32 μg/mL), cefazolin (MIC ≥64 μg/mL), cefoxitin (MIC ≥64 μg/mL), gentamicin (MIC ≥16 μg/mL), ciprofloxacin (MIC≥4 μg/mL), levofloxacin (MIC ≥8 μg/mL) and trimethoprim (MIC ≥320 μg/mL).

Genomic DNA of strain R17 was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Whole-genome sequencing was performed using Pacific Biosciences RSII platform (PacBio, Menlo Park, CA, USA) and Illumina Hiseq 2000 sequencer (Illumina, San Diego, CA, USA) as described before.[16] A chromosome of 5,124,258 bp in length with a GC content of 51.5% and a plasmid (pCFR17_1) of 206,820 bp in length with a GC content of 53.8% were assembled from the sequencing reads using SPAdes software (https://github.com/ablab/spades#sec5).[17,18]

Annotation with RAST server[19] revealed that the chromosome of strain R17 contained 4, 960 protein-coding genes, 42 repeat regions, 85 tRNAs and 25 rRNAs (eight complete 5S-23S-16S rRNA operons and one additional 5S rRNA). Two intact phage regions (one was 48 kb in length and another one was 44.8 kb in length) were predicted from the chromosome using PHASTER.[20] One AmpC-type beta-lactamase encoding gene, bla_{CMY-85}, was found in the chromosome of strain R17 using ResFinder 3.1[21] (Table 1). AmpC enzymes were reported to be involved in expanded-spectrum cephalosporins resistance and were found in the chromosomes of several members of *Enterobacterales* including *C. freundii*.[22] Up to date, 76.62% (272 of 355) *C. freundii* strains in NCBI Pathogen Detection database (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/) carried bla_{CMY} genes, indicating that bla_{CMY} genes were ubiquitous in *C. freundii*. To reveal the phylogenetic relationship between strain R17 and other *C. freundii* strains with published complete chromosome sequences, Average Nucleotide Identities (ANIs) were calculated based on MUMmer using JSpeciesWS.[23] Though strain R17 was environmental-derived, it showed high ANI values with the clinical or host-associated strains (Table S1), indicating its potential of being a pathogen. Notably, by searching against *C. freundii* locus/sequence definitions database (https://pubmlst.org/bigsdb?db=pubmlst_cfreundii_seqdef), we found that strain R17 could not be classified to any known STs, so it was assigned to a new ST, ST412.

Table 1 Distribution of ARGs in *C. freundii* strain R17

Resistance gene	Phenotype	Number of ARGs
Chromosome		1
bla_{CMY-85}	Beta-lactam resistance	1
pCFR17_1		12
aadA2	Aminoglycoside resistance	1
aac (3)-IId	Aminoglycoside resistance	1
bla_{DHA-1}	Beta-lactam resistance	1
bla_{TEM-1B}	Beta-lactam resistance	1
qnrB4	Fluoroquinolone resistance	1
mph (A)	Macrolide resistance	1
catA2	Phenicol resistance	1
sul1	Sulphonamide resistance	2
sul2	Sulphonamide resistance	1
tet (D)	Tetracycline resistance	1
dfrA12	Trimethoprim	1

Abbreviation: ARGs, antibiotic-resistance genes.

The plasmid typing using PlasmidFinder[24] indicated that pCFR17_1 was a Inc FII (Yp) type plasmid. A total of 12 ARGs were predicted in pCFR17_1 using ResFinder 3.1[21] (Table 1) and these genes were associated with resistance phenotypes of strain R17. By Blastn against NCBI database, we found that pCFR17_1 shared the highest similarity (99.99% identity and 73% coverage) with pCFR-e790 (GenBank accession number CP026241). Plasmid pCFR-e790 was identified in *C. freundii* strain CFNIH9 which was collected from wastewater of hospital internal pipes in USA.[25] These two plasmids shared the core backbone gene loci (Figure 1).

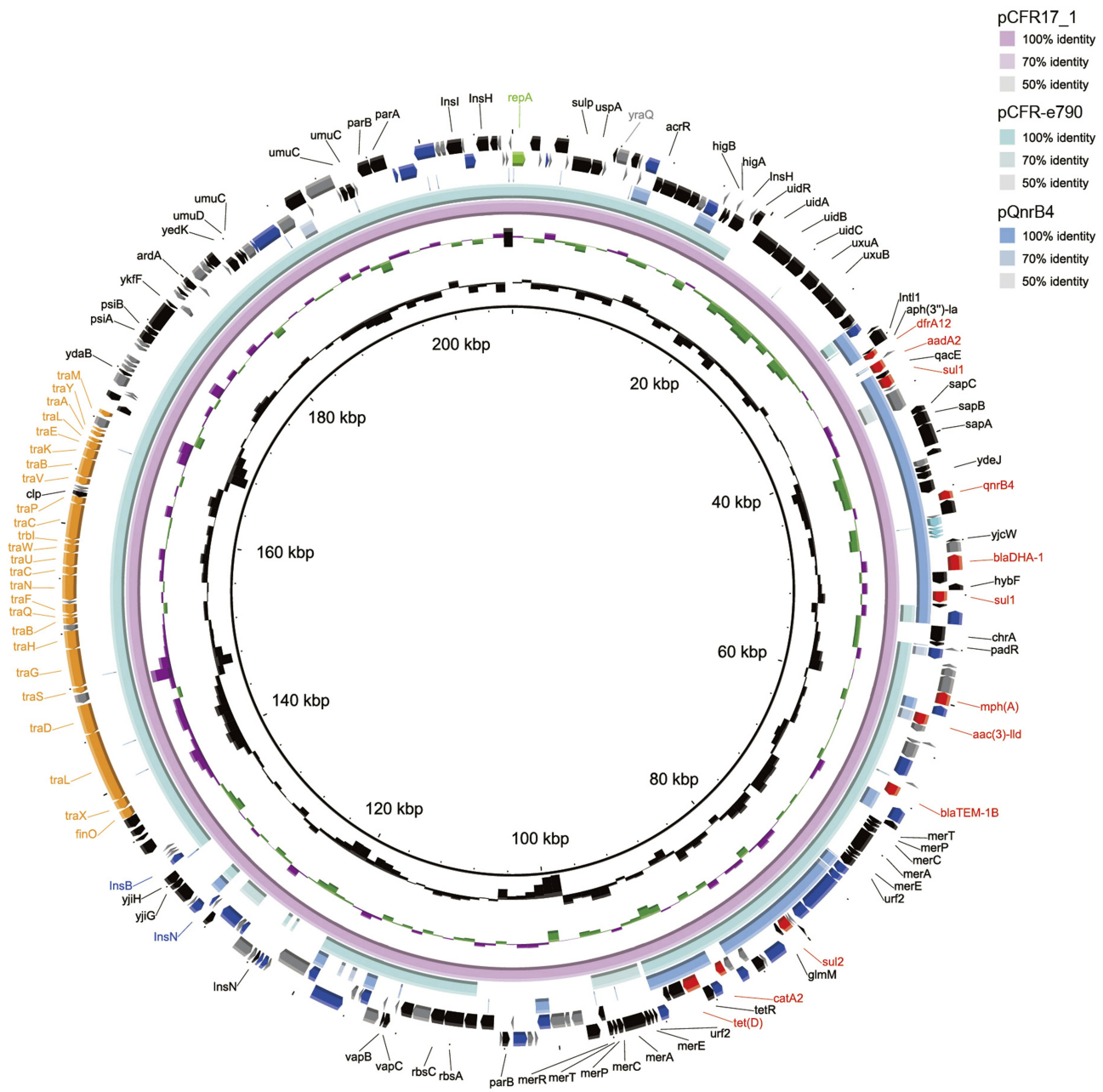

Figure 1 Circular comparison of plasmid pCFR17_1, pCFR-e790 and pQnrB4. The two outer circles showed the genes of pCFR17_1. The two inner circles were GC content and GC skew of pCFR17_1. The alignment of the three plasmids was performed and visualized by BLAST ring image generator (BRIG) software.

Lineal alignment between the two plasmids revealed three highly conserved regions that carried different resistance genes (Figure 2). Mobile elements set adjacent with these genes, indicating the transfer potential of them. One region in pCFR17_1 that contained *sul2, catA2* and *tet(D)* was reversed in pCFR-e790 with two insertion elements (*IS110* and *IS6*) located on both ends of this region, indicating there may be a re-organization mediated by these insertion elements. Sequences encoding for of several mercuric resistance genes were found to lie downstream of $bla_{\text{TEM-1B}}$ in both plasmids. This conserved sequence showed high similarity to TnAs3 which belonged to Tn3 composite transposon family. However, a ~24 kb region in pCFR17_1 was absent in pCFR-e790. And, this region exhibited a high similarity to pQnrB4 (GenBank accession number CP028537), a plasmid of *Enterobacter hormaechei* strain SCEH020042 which was a human-related strain isolated from Sichuan, China (Figure 1). Two insertion elements, *IS6* and *IS110*, located at each ends of this multidrug-resistant

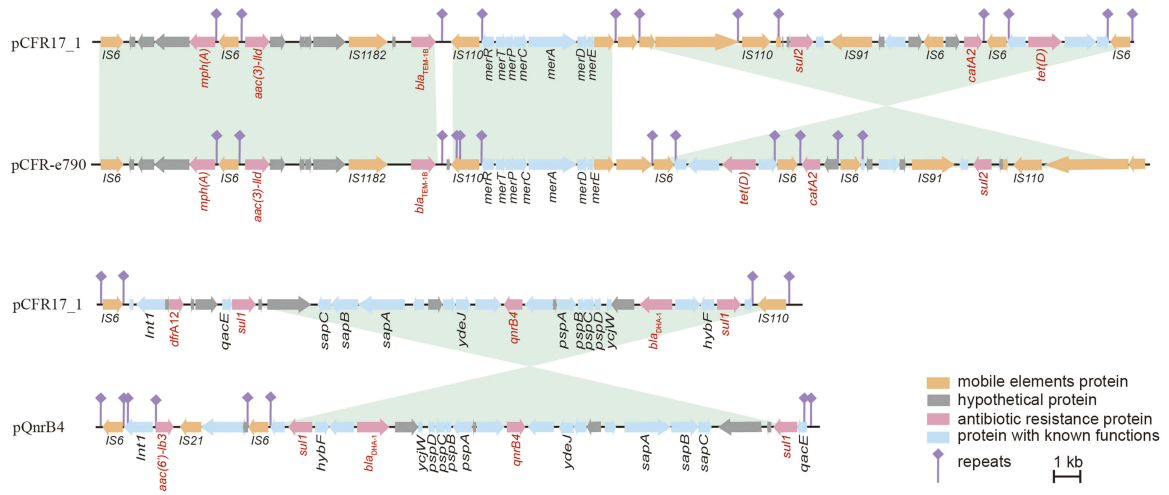

Figure 2 Lineal comparison of multidrug-resistant regions of pCFR17_1, pCFR-e790 and pQnrB4. Genes were portrayed by arrows and colored according to their functions.

region. Between these two insertion elements, there were five ARGs, *dfrA2, qnrB4,* bla_{DHA-1} and two *sul1* genes. In addition, an operon of phage shock proteins was also carried by this multidrug-resistant region between *qnrB4* and bla_{DHA-1} (Figure 2). Thus, our study is the first isolation of a novel multidrug-resistant environment-derived plasmid pCFR17_1 which showed similarity to both environment-derived plasmid pCFR-e790 and clinical-related plasmid pQnrB4. The emergence of this multidrug-resistant plasmid in strain R17 highlights the potential role of *C. freundii* as a vector for the dissemination of ARGs in aquatic environments.

In this study, we presented the sequences of the chromosome and the plasmid (pCFR17_1) of a multidrug-resistant *C. freundii* strain (R17). This is the first report of a novel ST (ST 412) *C. freundii* strain. The results of this work improved our understanding of the genetic context of *C. freundii* strains, as well as their antibiotic-resistance phenotypes and genotypes. Environment has been regarded as a potential reservoir of ARB with plasmids conferring resistance. The presence of ARGs, as well as mobile elements in strain R17, suggest that *C. freundii* may serve as potential source of resistance determinants for more virulent organisms. Thus, *C. freundii* and their plasmids should be monitored in the future.

Nucleotide sequence accession numbers: The nucleotide sequences of the chromosome and the plasmid (pCFR17_1) of *C. freundii* strain R17 have been deposited into DDBJ/EMBL/GenBank under accession number CP035276 and CP035277, respectively.

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Disclosure

The authors report no conflicts of interest in this work.

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