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Genome Note

Genomic scan of a healthcare-associated NDM-1-producing *Citrobacter freundii* ST18 isolated from a green sea turtle impacted by plastic pollution

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ABSTRACT

Background: Carbapenemase-producing *Citrobacter freundii* has been reported as a leading cause of healthcare-associated infections. Particularly, *C. freundii* belonging to the sequence type (ST) 18 is considered to be an emerging nosocomial clone.
Objectives: To report the genomic background and phylogenomic analysis of a multidrug-resistant NDM-1-producing *C. freundii* ST18 (strain CF135931) isolated from an endangered green sea turtle affected by plastic pollution in Brazil.
Methods: Genomic DNA was extracted and sequenced using the Illumina NextSeq platform. De novo assembly was performed by CLC Workbench, and in silico analysis accomplished by bioinformatics tools. For phylogenomic analysis, publicly available *C. freundii* (txid:546) genome assemblies were retrieved from the NCBI database.
Results: The genome size was calculated at 5 290 351 bp, comprising 5263 total genes, 4 rRNAs, 77 tRNAs, 11ncRNAs, and 176 pseudogenes. The strain belonged to *C. freundii* ST18, whereas resistome analysis predicted genes encoding resistance to β-lactams ($bla_{\text{NDM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CMY-117}}$, and $bla_{\text{TEM-1C}}$), aminoglycosides (*aph(3'')-Ib, aadA16, aph(3')-VI, aac(6')-Ib-cr,* and *aph(6)-Id*), quinolones (*aac(6')-Ib-cr*), macrolides (*mph(A)* and *erm(B)*), sulphonamides (*sul1* and *sul2*), tetracyclines (*tetA* and *tetD*), and trimethoprim (*dfrA27*). The phylogenomic analysis revealed that CF135931 strain is closely related to international human-associated ST18 clones producing NDM-1.
Conclusion: Genomic surveillance efforts are necessary for robust monitoring of the emergence of drug-resistant strains and WHO critical priority pathogens within a One Health framework. In this regard, this draft genome and associated data can improve understanding of dissemination dynamics of nosocomial clones of carbapenemase-producing *C. freundii* beyond hospital walls. In fact, the emergence of NDM-1-producing *C. freundii* of global ST18 in wildlife deserves considerable attention.

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1. Introduction

Citrobacter freundii is a Gram-negative bacterium of the *Enterobacterales* order considered an opportunistic causative agent of a broad spectrum of healthcare-associated infections, being frequently associated with the production of extended-spectrum β-lactamases and carbapenemases [1]. Of critical concern, there are increasing reports of NDM-1-producing *C. freundii* strains causing nosocomial outbreaks [1].

Although carbapenemase-producing *Enterobacterales* have emerged as serious cause of nosocomial infections worldwide, being classified as critical priority pathogens by the World Health

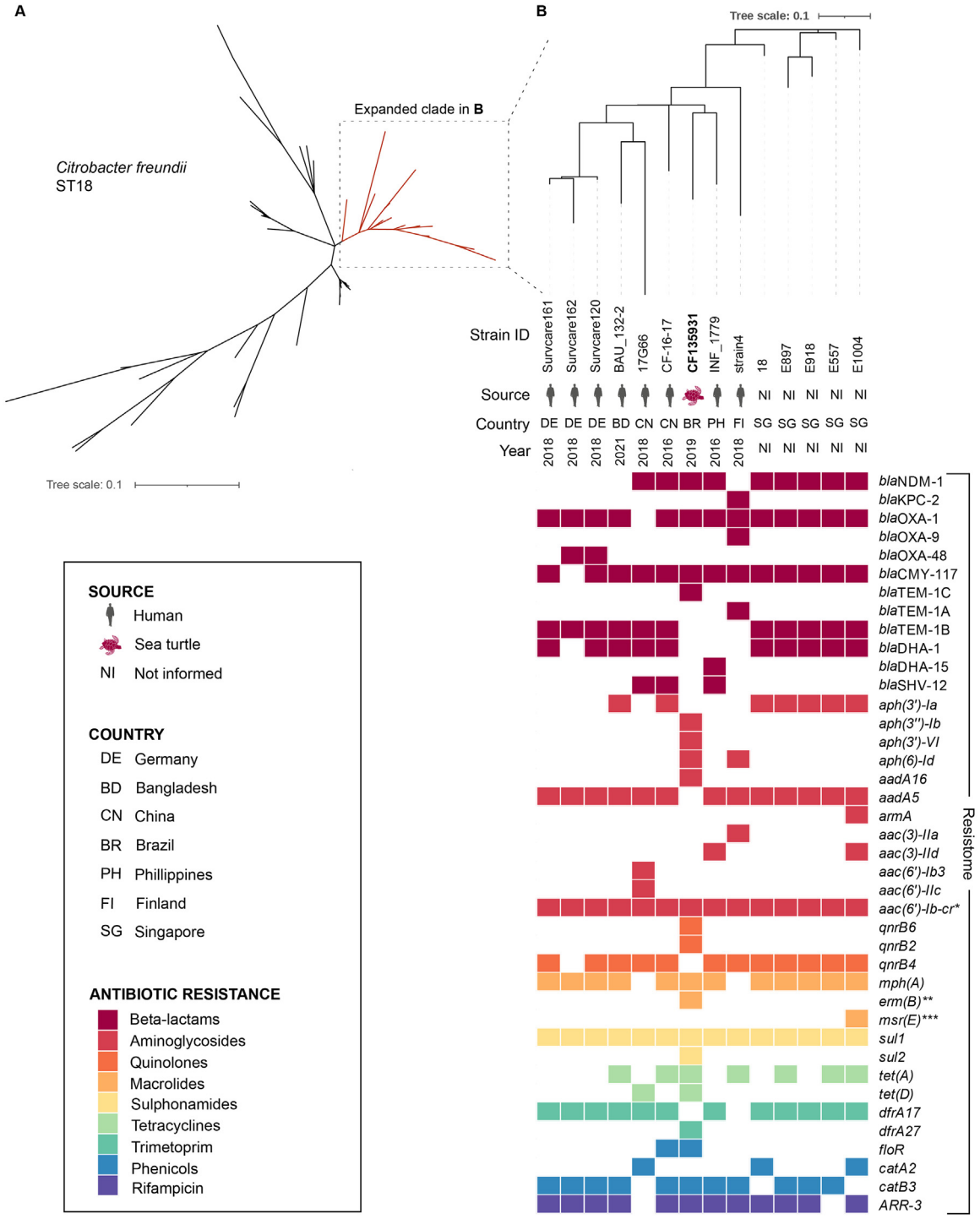

Fig. 1. SNP-based phylogenomic analysis of global *Citrobacter freundii* strains belonging to the international clone ST18. (A) Phylogenomic tree of *C. freundii* ST18 genome assemblies available at NCBI database. The SNP-based matrix of analysed genome assemblies is quoted in Supplementary Table S1. The clade containing the CF135931 strain is highlighted in red. (B) *C. freundii* ST18 genome assemblies closely related to the CF135931 strain are compared with resistome, source, country, and year of isolation.

Organization (WHO) [2], wildlife has been overlooked in the epidemiology of the carbapenem resistance problem [3], which poses a substantial ecological threat [1].

Herein, we present genomic data of an NDM-1-producing *C. freundii* belonging to the nosocomial sequence type (ST) 18 isolated from an endangered green sea turtle (*Chelonia mydas*) (https://www.worldwildlife.org/species/green-turtle) affected by plastic pollution in Brazil.

2. Methods

On 19 February 2019, a juvenile green turtle (*C. mydas*) was found stranded on a beach of São José (-27.60966; -48.62924), southern Brazil. The turtle was immediately transported to a sea turtle rehabilitation centre (Projeto Tamar – Florianópolis) for further clinical evaluation and treatment. Upon physical examination, the turtle displayed an injury on its head, signs of weakness and incoordination, anaemia, and dehydration. The turtle excreted nylon and other plastic fragments, indicating ingestion of marine plastic debris. As part of a genomic surveillance of WHO critical-priority pathogens colonizing Brazilian wildlife, a cloacal swab sample was collected, and microbiological analysis was performed as previously described [4]. In this regard, a Gram-negative bacterium (strain CF135931) was isolated, being identified as *C. freundii* by using MALDI-TOF/MS, and further confirmed by whole-genome sequencing (WGS) analysis. The strain CF135931 displayed a multidrug-resistant (MDR) profile to ertapenem, imipenem, meropenem, amoxicillin/clavulanic acid, cefoxitin, ceftiofur, cefotaxime, ceftazidime, ceftazidime/avibactam, cefepime, aztreonam, amikacin, gentamicin, nalidixic acid, ciprofloxacin, enrofloxacin, levofloxacin, trimethoprim/sulfamethoxazole, and tetracycline, as determined by the Kirby–Bauer method using Clinical and Laboratory Standards Institute (CLSI) breakpoints (Supplement M100, 31st ed.).

The genomic DNA of strain CF135931 was extracted using a PureLink™ Quick Gel Extraction kit (Life Technologies, Carlsbad, CA), and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA) was used to measure DNA concentration. Library preparation was performed using the Nextera DNA Flex kit (Illumina, San Diego, CA) and sequenced by using NextSeq550 platform paired-end reads (2 × 75 bp) (Illumina, San Diego, CA). Reads with a PHRED quality score below 20 were discarded, and adapters were trimmed using TrimGalore v.0.6.5 (https://github.com/FelixKrueger/TrimGalore). De novo genome assembly was performed with CLC Workbench v.12. Sequences were annotated using NCBI Prokaryotic Genome Annotation Pipeline version v.4.10 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

The genome size was calculated at 5 290 351 bp, comprising 5263 total genes, 4 rRNAs, 77 tRNAs, 11 ncRNAs, and 176 pseudogenes. Genomic typing of *C. freundii* was performed by MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/), plasmid replicons by PlasmidFinder 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/), and resistome by ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/), with a 95% threshold for gene identity and coverage.

For phylogenomic analysis, *C. freundii* (txid:546) genome assemblies were downloaded from the NCBI GenBank database (n = 1869) and screened using MLST v.2.23.0 (https://github.com/tseemann/mlst), which yielded 44 entries after removing contaminated genomes. The selected genomes were submitted to SNP analysis in the CSI Phylogeny software (https://cge.food.dtu.dk/services/CSIPhylogeny/), using the reference genome FDAARGOS_549 available in the NCBI RefSeq.

3. Results and discussion

The *C. freundii* CF135931 strain presented a wide resistome with genes encoding resistance to β-lactams ($bla_{\text{NDM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CMY-117}}$, and $bla_{\text{TEM-1C}}$), aminoglycosides (*aph(3'')-Ib, aadA16, aph(3')-VI, aac(6')-Ib-cr,* and *aph(6)-Id*), quinolones (*aac(6')-Ib-cr*, and *parC* [S80IN], *gyrA* [S83I], *gyrB* [E466D] mutations), macrolides (*mph(A)* and *erm(B)*), sulphonamides (*sul1* and *sul2*), tetracyclines (*tetA* and *tetD*), and trimethoprim (*dfrA27*). In addition, plasmid replicon types IncC, IncFIA, IncFIB, and IncR were predicted. The MLST analysis revealed that *C. freundii* CF135931 belonged to the ST18, which is considered an emerging clone of clinical concern [5]. The $bla_{\text{NDM-1}}$ gene was found to be located in the *trf-ble-*$bla_{\text{NDM-1}}$-IS*30* genetic context on a 6-kb contig displaying 100% identity and 99% coverage with pAB17 plasmid (Genbank accession no. MT002974.1), previously identified in a nosocomial *Acinetobacter baumannii* from Brazil (Supplementary Fig. S1).

The phylogenomic investigation confirmed that *C. freundii* CF135931 is genomically related (130–225 SNP differences) to human-associated ST18 lineages producing NDM-1 from China (2016 and 2018) and the Philippines (2016), and OXA-48- and KPC-2-positive clones identified in 2018, in Germany and Finland, respectively (Fig. 1; Supplementary Table S1). In this regard, the presence of these clinically relevant genes may be a strategy of the *C. freundii* ST18 lineage for a successful persistence in clinical environments.

These findings are remarkably important since they shed light on the appearance of healthcare-associated bacteria in an endangered marine animal [6]. Considering that carbapenemase-producing bacteria have been linked to contamination with land-based pollutants, the scientific community should pay more attention to the presence of MDR bacteria in marine animals affected by plastic pollution. Although our study does not confirm that plastic debris was the source of the origin of *C. freundii* strain CF135931, recent studies have suggested that the plastisphere can be a new pathway for MDR bacteria spread to the marine environment with uncertain consequences for their wildlife populations [7].

Our data may be useful for comparative genomic analyses of *C. freundii* belonging to the nosocomial clone ST18 strains that could emerge beyond hospital settings as a new threat for humans and nonhuman hosts [1,3,8]. In this sense, genomic databases available online allow bacterial sequence typing, tracking, and monitoring the spread of critically important antimicrobial-resistant bacterial lineages that cross the barriers of clinical environments [9].

Finally, since wildlife can be affected by *C. freundii* infections [8], this study also reinforces the urgent need to monitor the multidrug-resistant NDM-1-producing *C. freundii* that circulates among endangered wildlife populations.

Nucleotide sequence accession numbers: The whole genome nucleotide sequence of the *C. freundii* CF135931 isolate is available in the GenBank database under the accession number SRR25161878.

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Ethical approval: Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2024.01.006.

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