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Comprehensive whole genome sequencing with hybrid assembly of multi-drug resistant *Candida albicans* isolate causing cerebral abscess

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ABSTRACT

Comprehensive whole genome sequencing (WGS) with hybrid assembly of a multi-drug resistant (MDR) *Candida albicans* (CA) isolate causing cerebral abscess was performed using Illumina paired end and Oxford Nanopore long read technologies. The innovative technologies utilized here enabled us to resolve fragmented assemblies, and implement comprehensive and detailed genomic analyses involved in antifungal resistance of *Candida spp.* Functionally important genes (MDR1, CDR2 and SQN2) involved in antifungal resistance were identified and a phylogenetic analysis of the clinical isolate was performed. Additionally, our clinical isolate was found to share 4 single nucleotide polymorphisms with two other sequenced strains of MDR *C. auris* (381 and 386) including translation elongation factor EF1α and EF3, ATPase activity associated proteins, and the lysine tRNA ligase.

1. Introduction

1.1. Background

Multi-drug resistant (MDR) *Candida albicans* (CA) is a rare cause of invasive central nervous system infection. Risk factors for *Candida* infections include human immunodeficiency virus (HIV) infection, solid organ or bone marrow transplantation, malignancy, diabetes mellitus, prosthetic devices, central venous catheters, immunosuppressive agents and broad-spectrum antibiotic therapy (Kullberg and Arendrup, 2015; Petersen, 2010). Clinical manifestations range from mild mucosal infections to life-threatening disseminated infections. Interestingly, *Candida* cerebral abscess is a rare entity with <150 documented cases (Fennelly et al., 2013).

The most commonly prescribed anti-fungal used for the management of candidiasis is the azole fluconazole. The azole antifungals (*e.g.* fluconazole, voriconazole, posaconazole, itraconazole, isavuconazole) act to inhibit lanosterol 14-alpha-demethylase, an enzyme in the biosynthetic pathway needed to modify lanosterol to ergosterol, a key component of the fungal cell membrane. CA remains highly susceptible to fluconazole (resistance 0 – 5%) unlike *C. auris* (90%), an emerging global threat associated with multi-drug resistance and nosocomial outbreaks (Jeffery-Smith et al., 2018; Vallabhaneni et al., 2016; Whaley et al., 2016).

Here we report on an isolate of MDR CA (referred to as CA UCSD) causing cerebral abscess in a patient with uncontrolled HIV, performed comprehensive whole genome sequencing (WGS) with hybrid assembly of the isolate enabling a more detailed phylogenetic analysis, attempted to identify novel candidate resistance genes, and performed a comparative analysis to other strains of CA and MDR *C. auris* (Miller et al., 2017).

1.2. Source case

In June 2017, a 45-year-old woman with a history of uncontrolled HIV/AIDS (CD4 count 27 cells/uL, CD4% 3%, HIV RNA viral load

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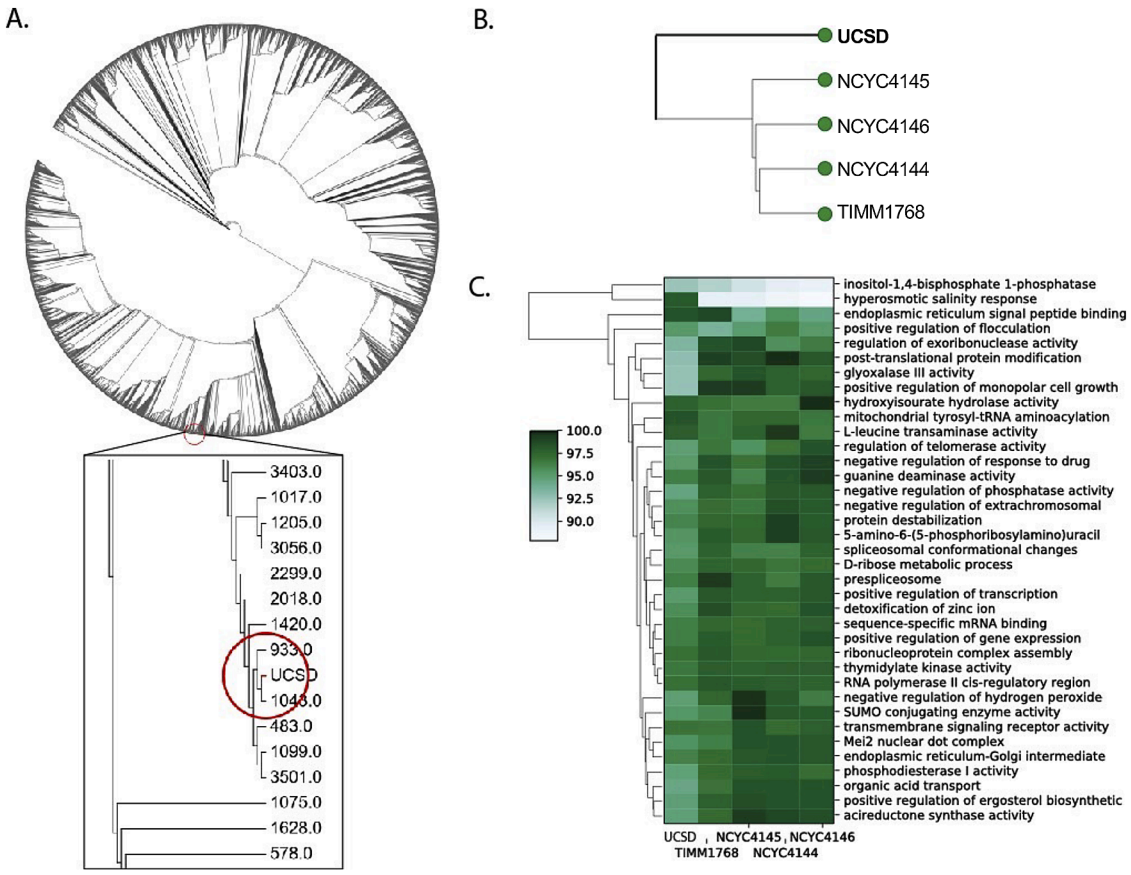


Fig. 1. (A) Phylogenetic tree based on concatenated MLST sequences for all 3569 sequences available at the *Candida* MLST database. (B) Hierarchical clustering tree of *C. albicans* core genome (defined as the set of 2688 genes with 98% or greater nucleic acid identity across all five *C. albicans* strains). (C) A heatmap of functions with the least conservation across all five *C. albicans* strains.

27,820 copy/mL), cryptococcal meningitis (stable serum cryptococcal Ag titer of 1:20), recurrent oropharyngeal candidiasis, severe periodontal disease, uncontrolled type 2 diabetes mellitus, methamphetamine use and poor medication compliance presented to the hospital with a one-week history of nausea, vomiting, headache and confusion. Physical examination was notable for cachexia, severe periodontal disease, and extensive raised, creamy white patches along the tongue and posterior oropharynx. Cardiovascular exam did not reveal evidence to support infective endocarditis. Cranial and peripheral nerve function were grossly intact.

Brain computed tomography (CT) and magnetic resonance imaging demonstrated a multi-loculated cystic lesion in the left parietal lobe concerning for intracranial abscess secondary to septic emboli (Supplementary Figs. 1 and 2). CT of the chest/abdomen/pelvis revealed no septic emboli, and transesophageal echocardiography had no evidence of valvular vegetations or perivalvular abscess. Ultimately, a left sided craniotomy with subdural drainage of intra-parenchymal abscess was performed.

Intraoperative pathology and cultures were consistent with *Candida spp.* and a heterogenous population of azole resistant CA respectively (Supplementary Table 1). All three clinical CA isolates identified from culture with heavy growth were resistant to fluconazole per broth microdilution antimicrobial susceptibility testing performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines, while one isolate was nearly pan-azole resistant (isavuconazole MIC = 0.06 μg/mL). Subsequent cerebrospinal fluid analysis revealed RBC 56 mm^3, WBC 74 mm^3 (neutrophils 42%, lymphocytes 49%, eosinophils 1%, macrophages 8%), glucose 62 mg/dL, total protein 95 mg/dL, negative cryptococcal Ag, negative *Mycobacterium tuberculosis* complex PCR, and bacterial, fungal and acid-fast bacilli cultures with no growth. The patient was started on intravenous amphotericin B and oral flucytosine for CA cerebral abscess presumed secondary to candidemia, endocarditis and septic emboli caused by poor dentition and recurrent oropharyngeal candidiasis.

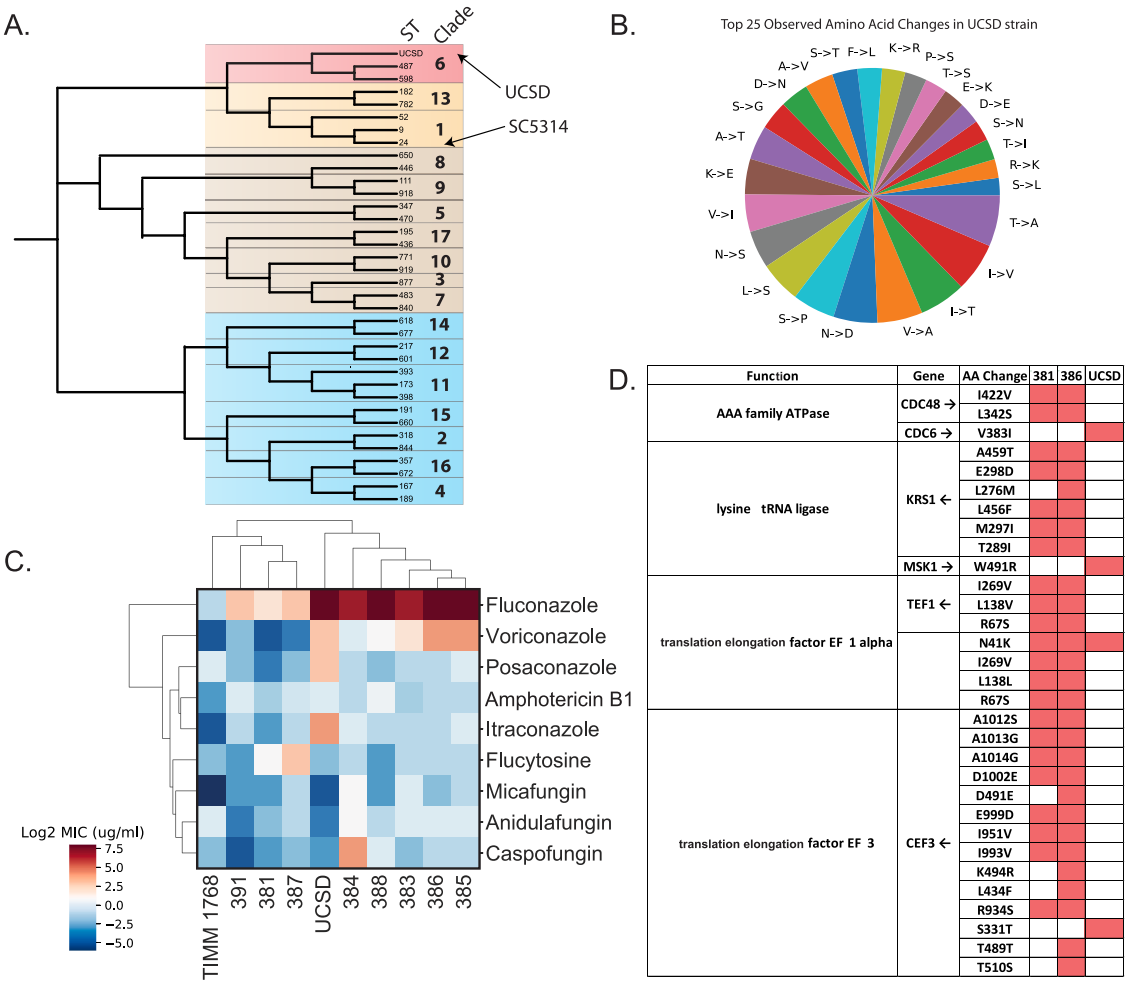
2. Materials and methods

2.1. Genome sequencing and assembly

WGS of the CA UCSD isolate was performed using Illumina paired-end and Oxford Nanopore technologies (Jolley and Maiden, 2010; Katoh and Standley, 2013; Kolmogorov et al., 2018; Koren et al., 2017; Panthee et al., 2018; Price et al., 2010; Proux-Wéra et al., 2012; Salmela and Rivals, 2014). Oxford Nanopore long reads were prepared using the RAD004 kit per manufacturer's protocol (Oxford Nanopore Technologies, UK). Live reads were disabled and bases were called using Guppy v3.2.1 + 334123b. We obtained 259k reads from MinION (mean length 2.5 kb, 45X coverage) and 3.4 M reads using Illumina PE150 (mean length 150 bp, 35X coverage). Genome assembly was performed using Flye 2.3.3 assembler (Kolmogorov et al., 2018). The high quality MinION long reads were filtered using Filtlong followed by self-correction and trimming using canu 1.7 (Koren et al., 2017). The short reads were then used for error correction of the long trimmed reads using LoRDEC (Salmela and Rivals, 2014). The final assembly of the corrected reads was then performed with the Flye genome assembler.

2.2. Comparative genomic analyses

Phylogenetic comparisons were performed by concatenation of the nucleotide sequences for the alleles and aligned using mafft followed by



Function	Gene	AA Change	381	386	UCSD
AAA family ATPase	CDC48 →	I422V			
		L342S			
	CDC6 →	V383I			
lysine tRNA ligase	KRS1 ←	A459T			
		E298D			
		L276M			
		L456F			
		M297I			
		T289I			
	MSK1 →	W491R			
translation elongation factor EF 1 alpha	TEF1 ←	I269V			
		L138V			
		R67S			
		N41K			
		I269V			
		L138L			
		R67S			
translation elongation factor EF 3	CEF3 ←	A1012S			
		A1013G			
		A1014G			
		D1002E			
		D491E			
		E999D			
		I951V			
		I993V			
		K494R			
		L434F			
		R934S			
		S331T			
		T489T			
		T510S			

Fig. 2. (A) MLST dendrogram of *C. albicans* UCSD and selected sequence types spanning the 17 clades of *C. albicans* (Odds et al., 2007). CA UCSD was found to cluster with members of clade 6 according to a concatenated alignment of MLST sequences. B) Top 25 AA changes observed in CA UCSD relative to CA SC 5314. T->A mutations were the top observed mutation types (79, 3.5%) followed by I->V (76, 3.4%) and I->T (72, 3.2%). C) Measured MICs for nine antifungal drugs across 10 *Candida* strains. Strains TIMM1768 and UCSD are *C. albicans*, strain 391 is *C. duobushaemulonii*, and the remaining strains are *C. auris* isolates collected by the CDC AR Isolate Bank. D) SNPs shared between *C. albicans* UCSD and two *C. auris* strains. SNPs leading to amino acid coding changes across the three strains.

phylogenetic tree building with fastree (Fig. 1A). Next, the nucleic acid similarity of genes and point mutations in CA UCSD relative to CA SC5314 (NCBI ID: GCA_000182965.3) were determined using BLASTn and breseq respectively. The 5 strains of *C. albicans* were compared to this reference strain by running BLASTn against the nucleotide sequences of all ORFs annotated on the genome of CA SC5315 (Fig. 1B). Percentage identities for all genes were calculated and the top 50 genes with the lowest level of percentage identity were displayed in Fig. 1C with their functions. We also analyzed and compared two *C. auris* strains (381 and 386) selected from the CDCs databank to CA UCSD using breseq (Deatherage and Barrick, 2014).

3. Results and discussion

The assembled genome of 14.5 megabase-pairs indicated a diploid state (Selmecki et al., 2015). With 6223 ORFs identified, the multi-locus sequence typing (MLST) allele profile was novel (AAT1a: 3, AAC: 3, ADP1:52, MPIb: 9, SYA1:26, VPS13:24, ZWF1b: 12). Phylogenetic analysis of CA UCSD showed greatest similarity to MLST sequence types 933 (sputum isolate from Taiwan) and 1043 (blood isolate from Portugal) (Fig. 1). These sequence types are members of Clade 6 known to be enriched with strains exhibiting reduced fluconazole sensitivity (Fig. 2A) (McManus and Coleman, 2014; Odds et al., 2007).

CA UCSD had 14,628 mutations relative to reference strain CA SC 5314, of which 7105 were coding changes within annotated ORFs and 7523 mutations in intergenic regions. Among coding changes, 2255 single nucleotide polymorphisms (SNPs) resulted in non-synonymous amino acid coding changes across 1259 unique genes grouped into 690 distinct annotated functions (Supplementary Data File 1). The function with most mutations (outside of hypothetical proteins (918 mutations)) were for Als5p (23 mutations) and Als6p (21 mutations). The ALS genes of *C. albicans* encode a family of cell-surface glycoproteins and are thus likely under selective pressure due to interactions with the environment and immune system. Overall, there were 175 distinct amino acid (AA) coding changes across the 2255 non-synonymous mutations. The top 25 mutation types were responsible for 1219 of these mutations (54% of all non-synonymous mutations) (Fig. 2B). Threonine to Alanine (T->*A*) mutations were the top observed mutation types (79, 3.5%) followed by Isoleucine to Valine (I->*V*) (76, 3.4%) and Isoleucine to Threonine (I->*T*) (72, 3.2%). We perfomed a literature search on 32 mutations across 10 genes known to be involved in azole resistance (Whaley et al., 2016). Some of the SNPs observed in CA UCSD have been observed in the literature such as MRR1 SNPs V341E and V1020Q. However, these two SNPs were not reported as gain of function mutations in resistant strains. A further 27 mutations were seen in regions upstream of these genes potentially affecting their expression, including C->A 66 base pair (bp) upstream of MDR1, a known binding site of MRR1 (Schubert et al., 2011). We also analyzed and compared the

Table 1
Genes mutated in CA UCSD with known roles in azole resistance.

Mutated Gene	Product	Mutation	Chr. Position	Mutation
GSC1 ←	1,3-beta-glucan synthase	I862I (ATC→ATT)	509,077	G→A
	1,3-beta-glucan synthase	A153A (GCT→GCA)	511,204	A→T
GSL2 ←	Gsl2p	E689E (GAA→GAG)	195,028	T→C
ERG11 ←	sterol 14-demethylase	L480L (TTA→TTG)	148,262	T→C
MRR1 →	Mrr1p	L248V (TTA→GTA)	1330,004	T→G
	Mrr1p	A324A (GCC→GCT)	1330,234	C→T
	Mrr1p	L333L (CTT→CTC)	1330,261	T→C
	Mrr1p	V341E (GTA→GAA)	1330,284	T→A
	Mrr1p	T378T (ACT→ACA)	1330,396	T→A
	Mrr1p	K479K (AAG→AAA)	1330,699	G→A
	Mrr1p	coding (1773-1774/3327 nt)	1331,035	2 bp→TT
	Mrr1p	L592L (CTC→CTA)	1331,038	C→A
	Mrr1p	S594S (TCT→TCC)	1331,044	T→C
	Mrr1p	V597V (GTC→GTT)	1331,053	C→T
	Mrr1p	S602S (TCT→TCA)	1331,068	T→A
	Mrr1p	G604G (GGT→GGA)	1331,074	T→A
	Mrr1p	E608E (GAA→GAG)	1331,086	A→G
	Mrr1p	N617N (AAT→AAC)	1331,113	T→C
	Mrr1p	E1020Q (GAA→CAA)	1332,320	G→C
CDR1 →	ATP-binding cassette multidrug transporter	G426G (GGT→GGA)	1147,322	T→A
	ATP-binding cassette multidrug transporter	N1220N (AAT→AAC)	1149,704	T→C
CDR2 →	Cdr2p	S632L (CTG→TTG)	1049,780	C→T
	Cdr2p	R683K (AGA→AAA)	1049,934	G→A
	Cdr2p	L723L (CTT→CTC)	1050,055	T→C
TAC1 ←	Tac1p	Q877Q (CAA→CAG)	416,715	T→C
	Tac1p	T863T (ACC→ACT)	416,757	G→A
MSH2 ←	mismatch repair ATPase	I714I (ATT→ATC)	1576,063	A→G
PUP1 →	proteasome core particle subunit beta 2	A21A (GCT→GCC)	2195,688	T→C
SNQ2 ←	ATP-binding cassette transporter	A1104A (GCT→GCC)	833,471	A→G
	ATP-binding cassette transporter	S800L (CTG→CTA)	834,383	C→T
	ATP-binding cassette transporter	Q702Q (CAG→CAA)	834,677	C→T

MICs and SNPs of *C. auris* strains from the CDC AR Isolate Bank to CA UCSD (Fig. 2C and 2D).

Despite antifungal therapy and/or surgical intervention, outcomes for *Candida* cerebral abscess remain poor with morbidity and mortality rates of up to ~70% (Fennelly et al., 2013). Further complicating the matter in our patient was the presence of nearly pan-azole resistant CA infection, severely limiting oral step-down therapeutic options. Rarely seen in the clinical setting, the extreme resistance observed likely emerged as the result of prior azole exposure for management of oropharyngeal candidiasis and cryptococcal meningitis.

To examine the genetic basis for azole resistance, we compared CA UCSD with pan-azole sensitive CA TIMM1768 and eight *C. auris* strains exhibiting various levels of antifungal resistance. CA UCSD was found to exhibit higher azole resistance relative to any of the other strains (Fig. 2C). We identified mutations in 10 different genes known to confer azole resistance including three proteins related to efflux pump activity; MDR1, CDR2 and SQN2 (Table 1) (Rad et al., 2016; Sanglard et al., 1997; Sanguinetti et al., 2006).

MDR1, a Major Facilitator Superfamily (MFS) transporter, is frequently overexpressed in azole resistant isolates. MRR1 is a zinc-finger transcription factor that controls expression of the MDR1 gene. Interestingly, multiple studies have identified gain-of-function point mutations in both clinical and *in vitro* generated azole-resistant *Candida* that result in a constitutively active MRR1 protein and subsequent upregulation of MDR1 (Dunkel et al., 2008). In addition to coding changes within the protein, we identified mutations in promoter regions of these genes. Increased expression of ABC transporter genes, CDR2 and SQN2, have also been implicated in azole resistance.

Interestingly, two of the six coding changes observed in genes known to be involved in azole resistance consisted of serine (CTG) to leucine coding changes (Cdr2p, S632L and SNQ2, S800L) (Sárkány et al., 2014). These two ATP-binding cassettes (ABC) transporters have been shown to mediate antifungal resistance *in vivo* through drug efflux. *C. albicans* is one of the CTG-clade species that exhibits sense-to-sense codon reassignment leading to ambiguity of the CTG codon under stress and incorporation of either serine or leucine. This mechanism has been shown to generate ambiguous proteins thus expanding the possible proteome of these species. However, in this case it may be that exclusively coding for leucine ensures translation to a protein form more active in ABC transport processes and aids in azole resistance. Besides these 2 coding changes known to be involved in azole resistance we observed 25 more serine to leucine mutations in strain UCSD including in the genes Rim8p (involved in pH response), Sap99p (secreted aspartyl protease) and CTA4 (oleate-activated transcription factor).

Finally, to identify potentially novel mutations associated with azole resistance we searched for shared SNPs between CDC *C. auris* isolates displaying azole resistance and CA UCSD. We found that different mutations in genes encoding for translation elongation factor EF1α and EF3 along with ATPase activity associated proteins, and the lysine tRNA ligase were shared amongst these isolates (Fig. 2D). Thus, further studies examining these mutations and their potential impact on azole resistance in *Candida* spp. are warranted.

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Disclaimer

None

Author contributions

MK provided source case, clinical isolate for whole genome sequencing analysis, assisted in preliminary comparative genomic analyses, and wrote the preliminary manuscript. AC assisted with comparative genomic analyses and identification of key genes involved in azole resistance. RS assisted with DNA extraction of clinical isolate and whole genome sequencing. TCSM assisted with review of source case and editing of manuscript. The project was conceived and developed by MK,

BP, VN and JMM. JMM conducted detailed comparative genomic analyses, and generated tables and figures. All authors (MK, AC, RS, TCM, BP, VN and JMM) contributed to editing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

This whole-genome project has been deposited at NCBI BioProject under the accession PRJNA625339 and genome accession JABAHU000000000.

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None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100180.

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