Computational and Structural Biotechnology Journal 21 (2023) 3885–3893

Contents lists available at ScienceDirect

Computational and Structural Biotechnology Journal

journal homepage: www.elsevier.com/locate/csbj

Research article

Molecular epidemiology and genomic characterization of a plasmid-mediated *mcr-10* and *bla*$_{NDM-1}$ co-harboring multidrug-resistant *Enterobacter asburiae*

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ARTICLE INFO

Keywords:
mcr-10
bla$_{NDM-1}$
Lipid A
Colistin resistance
Enterobacter asburiae

ABSTRACT

Colistin is considered as one of the last-resort antimicrobial agents for treating multidrug-resistant bacterial infections. Multidrug-resistant *E. asburiae* has been increasingly isolated from clinical patients, which posed a great challenge for antibacterial treatment. This study aimed to report a *mcr-10* and *bla*$_{NDM-1}$ co-carrying *E. asburiae* clinical isolate 5549 conferred a high-level resistance against colistin. Antibiotic susceptibility testing was performed using the microdilution broth method. Transferability of *mcr-10* and *bla*$_{NDM-1}$-carrying plasmids were investigated by conjugation experiments. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to identify modifications in lipid A. Whole genome sequencing and phylogenetic analysis between strain 5549 and a total of 301 *E. asburiae* genomes retrieved from NCBI database were performed. The genetic characteristics of *mcr-10* and *bla*$_{NDM-1}$-bearing plasmids were also analyzed. Our study indicated that strain 5549 showed extensively antibiotic-resistant trait, including colistin and carbapenem resistance. The *mcr-10* and *bla*$_{NDM-1}$ were carried by IncFIB/IncFII type p5549_mcr-10 (159417 bp) and IncN type p5549_NDM-1 (63489 bp), respectively. Conjugation assays identified that only the *bla*$_{NDM-1}$-carrying plasmid could be successfully transferred to *E. coli* J53. Interestingly, *mcr-10* did not mediate colistin resistance when it was cloned into *E. coli* DH5α. Mass spectrometry analysis showed the lipid A palmitoylation of the C-lacyl-oxo-acyl chain to the chemical structure of lipid A at *m/z* 2063 in strain 5549. In summary, this study is the first to report a *mcr-10* and *bla*$_{NDM-1}$ co-occurrence *E. asburiae* recovered from China. Our investigation revealed the distribution of different clonal lineage of *E. asburiae* with epidemiology perspective and the underlying mechanisms of colistin resistance. Active surveillance is necessary to control the further dissemination of multidrug-resistant *E. asburiae*.

1. Introduction

Colistin is one of the last-resort antibiotics in clinical treatment since the emergence of CRE (carbapenem-resistant Enterobacteriaceae), especially CRECC (carbapenem-resistant *Enterobacter cloacae* complex) has seriously compromised the effectiveness of the available antimicrobial agents [1]. According to the most recent data from China Antimicrobial Surveillance Network (CHINET), the rate of carbapenem resistance for *E. cloacae* complex (ECC) was 1% in 2007 and rapidly increased to 10% in 2021. Diverse carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, and *bla*$_{IPM}$) were identified in CRECC, of which the *bla*$_{NDM}$ gene was the most dominant in China [2]. At the same time, colistin-resistant CRECC strains have been found in hospitalized patients, which was considered a major public health threat worldwide due to the limited alternative therapeutic choices [3]. In *Enterobacter* spp., colistin resistance is conferred through modification of lipid A, the membrane anchor of lipopolysaccharide (LPS), which reduces colistin binding affinity [4]. Molecular mechanisms of colistin resistance can be attributed to chromosomal mutations and

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https://doi.org/10.1016/j.csbj.2023.08.004
Received 6 February 2023; Received in revised form 29 July 2023; Accepted 4 August 2023
Available online 6 August 2023

plasmid-mediated mobile colistin resistance genes (*mcr*). The first *mcr* gene, *mcr-1* was identified from *Escherichia coli* in 2016 in China [5], which variants have also been found in *Enterobacter* spp., including *mcr-1* [6], *mcr-3* [7], *mcr-4* [8], *mcr-5* [9], *mcr-9* [10], and *mcr-10* [11]. The modification of LPS via two-component system (TCS) was a common strategy to reduce colistin susceptibility in Gram-negative bacteria [4]. Although the mechanisms of colistin resistance in *Enterobacter* spp. have not been completely explored, various types of lipid A modifications have been observed. These lipid A modifications in ECC include a range of structural changes, notably the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (PEtN) [12], as well as hydroxylation and palmitoylation of lipid A moiety [13].

E. asburiae is a gram-negative bacterium belonging to the ECC, which was first described and identified in 1986 [14]. Its strains have been isolated from a variety of human, environment, and plants as opportunistic pathogens [15,16]. *E. asburiae* is widely isolated throughout the United States due to the horizontal transfer of $bla_{\text{IMI-2}}$ carbapenemase gene between clinical and river environments [17]. A study collected *mcr-9/10* positive *E. asburiae* showed that sequence type (ST) 484 *mcr-9*-carrying *E. asburiae* strains were dominant (29.4%, 10/34) [18]. Besides, ST252 *E. asburiae* was reported as the predominant reservoir of a new transferable quinolone resistance *qnrE* family allele gene qnr_{Eas1} [19]. Nevertheless, so far, no *mcr-10* and $bla_{\text{NDM-1}}$ co-carrying ST252 *E. asburiae* has been reported. Since *mcr-10* gene was recently identified on a IncFIA-type plasmid from *E. roggenkampii* [11], other *mcr-10*-bearing *Enterobacter* spp. strains were found successively in different types of plasmids (IncFII, IncFIB, IncFIB, and IncFIA) [11,20,21] and different species, including *E. cloacae*, *E. kobei*, *E. asburiae*, *E. ludwigii*, and *E. hormaechei* [22]. Compared to *mcr-10*, *mcr-9* has a higher isolation rate from conserved plasmid types (IncHI2/2 A) in ECC strains and usually coexisted with carbapenemase genes [18]. Moreover, in a recent report, *mcr-9* was co-transferred with $bla_{\text{NDM-1}}$ among ECC strains, although transconjugants did not show resistance to colistin [23]. For *mcr-10*, it has the highest nucleotide identity with *mcr-9*, and both mediated low-level of resistance to colistin [11,24]. Previous study has confirmed the essential role of a two-component system QseB/C in *mcr-9* inducible expression [25], but whether there is a driving force behind colistin resistance for *mcr-10* is still unknown.

The objective of this study was to report the genomic characteristics of a multidrug-resistant *E. asburiae* strain co-carrying plasmid-borne *mcr-10* and $bla_{\text{NDM-1}}$ genes in China, as well as to investigate the underlying mechanisms of colistin resistance in ECC. In addition, we investigated the genomic epidemiology of ST252 *E. asburiae* in a global context and emphasized the need to restrict the spread of the mobile colistin and carbapenem resistance genes in ECC.

2. Materials and methods

2.1. Bacterial isolate

A 45-year-old woman was diagnosed with bronchial granuloma at a tertiary hospital in China in 2019 while suffering from coughing and shortness of breath after a vehicle accident. The strain 5549 was cultured from a urine sample during her hospitalization. Identification of the isolate was carried out using VITEK (bioMérieux, Marcy-l'Étoile, France) and Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker, Billerica, MA, United States). The study was approved by the Ethical Committee of the Fourth Affiliated Hospital, Zhejiang University School of Medicine, China (K2022112). No identifiable patient information was obtained, this study focused mainly on bacteria and did not involve intervention in the patient.

2.2. Antimicrobial susceptibility testing

According to the Clinical and Laboratory Standards Institute (CLSI) guidelines [26], the broth microdilution method was used to evaluate the minimum inhibitory concentrations (MICs) of 18 antibiotics, including aztreonam (ATM), ceftazidime (CAZ), cefotaxime (CTX), ciprofloxacin (CIP), cefepime (FEP), cefoxitin (FOX), levofloxacin (LVX), amikacin (AMK), gentamicin (GEN), meropenem (MEM), imipenem (IPM), tigecycline (TGC), colistin (CST), trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), ampicillin-sulbactam (SAM) and ceftazidime-avibactam (CAS). Except for colistin and tigecycline resistance clinical breakpoints, which were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (https://eucast.org/clinical_breakpoints/), the antimicrobial susceptibility data for other antibiotics were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The quality control strain used in this study was *E. coli* ATCC 25922.

2.3. Conjugation experiments

The conjugation experiment was performed using the azide-resistant *E. coli* J53 as the recipient and strain 5549 as the donor. Donor and recipient strains (2 mL each) were cultured overnight shaking at 37 °C and mixed together at a donor-recipient ratio of 1:3. The mixed strains were collected by centrifugation for 2 min at 5000 rpm, resuspended in 100 μL LB broth and cultured on filters at 37 °C bacteria incubator overnight [27]. Potential contained $bla_{\text{NDM-1}}$ gene transconjugants were selected on Mueller-Hinton agar plates containing 200 mg/L azide and 4 mg/L imipenem. According to previous studies [11,20], *mcr-10* had a weak role in colistin resistance and might not confer colistin resistance phenotype. We selected *mcr-10* potential transconjugants with 200 mg/L azide and 1 mg/L colistin. oriTfinder online tool (https://tool-mml.sjtu.edu.cn/oriTfinder/oriTfinder.html) was used to inspect for the origins of transfer (oriTs) and other conjugation gene modules.

2.4. mcr-10 gene cloning

The *mcr-10* gene containing its promoter region was amplified from strain 5549. The purified PCR fragment was inserted into the constructed pCR2.1-Hyg vector with hygromycin-resistance from the *Hind*III site using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) resulting in pCR2.1-Hyg-mcr-10. The recombinant was introduced into the *E. coli* DH5α strain by chemical transformation. Transformants were selected on Mueller-Hinton agar plates containing 40 mg/L ampicillin and colonies on plates were confirmed by PCR using and Sanger sequencing.

2.5. S1-PFGE and Southern hybridization

S1-PFGE and Southern hybridization were conducted to determine the sizes of *mcr-10* and $bla_{\text{NDM-1}}$-carrying plasmids in strain 5549 and the transconjugants. The genomic DNA was digested with S1 nuclease (Takara Biotechnology, Dalian, China) and produced in Lonza Bioscience SeaKem® Gold Agarose, then electrophoresis by the CHEF-Mapper PFGE system (Bio-Rad) under the conditions of 14 °C, 6 V/cm, running for 16 h, 120° pulse angle and pulse times from 2.16 s to 63.8 s. Plasmid DNA fragments were hybridized with digoxigenin-labeled $bla_{\text{NDM-1}}$ or *mcr-10* specific probes after being transferred to nylon membranes and detected using DIG High Prime DNA Labeling and Detection Starter KitI (Roche, Mannheim, Germany).

2.6. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed to measure the expression of genes in known two-component system (PhoP, PhoQ, PmrA, and PmrB) and MgrB inactivation. Total RNA was extracted by the RNApure Bacteria kit (CWBIO, China). Reverse transcription reactions and qRT-PCR were performed by Evo M-MLV RT Mix Kit (Accurate Biology, China) and NovoStart® SYBR qPCR SuperMix Plus kit

(Novoprotein, China). The relative expression levels in comparison with the reference strain *E. asburiae* ATCC 35953 were calculated by the $2^{-\Delta\Delta CT}$method. The primers used here were listed in Table S1.

2.7. Isolation of lipid A and mass spectrometry

Lipid A were extracted from *E. asburiae* ATCC 35953 and strain 5549 according to previous description [28]. Briefly, overnight a single colony was grown in 10 mL LB broth (37 °C, 200 rpm), collected and lyophilized. The freeze-dried bacteria (10 mg) were suspended in a 400 μL mixture (isobutyric acid and 1 M hydroxide 5:3, v/v) and heated for 2 h at 100 °C under stirring. The mixture was cooled on the ice and centrifuged at 2000 g for 15 min. The supernatant was supplemented with an equivalent volume of water (1:1, v/v) and lyophilized overnight. The crude lipid A was subjected to two consecutive washes with 400 μL methanol followed by centrifugation at 2000 g, 15 min. Finally, the insoluble lipid A fraction was extracted using a mixture consisting of chloroform, methanol, and water (3:1.5:0.25, v/v) in a volume of 100 μL. Image data of lipid A was collected in the negative ion mode using a reflectron geometry MALDI TOF-TOF mass spectrometer.

2.8. Whole-genome sequencing and bioinformatics analysis

Following the manufacturer's protocols, the genomic DNA of strain 5549 was extracted by the QIAamp DNA MiniKit (Qiagen, Valencia, CA, United States). The prepared genomic DNA was subjected to the Illumina NovaSeq 6000 (Illumina, San Diego, CA, United States) and Oxford Nanopore GridION (Nanopore, Oxford, UK) sequencing. The sequencing data of both short and long reads were assembled using Unicycler v0.5.0 [29]. The annotation of the genome was conducted by the Prokaryotic Genome Annotation Pipeline (PGAP) [30]. Average nucleotide identity (ANI) was performed on the JSpeciesWS online tool (https://jspecies.ribohost.com/jspeciesws/) to identify the specific species of ECC for strain 5549. *In silico* multilocus sequence typing (MLST) analysis was performed using mlst (https://github.com/tseemann/mlst). Antimicrobial resistance genes, virulence genes, and plasmid replicons were identified using the ABRicate program (https://github.com/tseemann/abricate), which bundled with ResFinder [31], VFDB [32], and PlasmidFinder [33]. A circular comparison of different plasmids and plasmid maps were created using BLAST Ring Image Generator (BRIG) [34]. Genetic context of *mcr-10*, bla_{NDM-1} and linear comparison were visualized by Easyfig tool (http://mjsull.github.io/Easyfig/).

2.9. Single-nucleotide polymorphisms and phylogenetic analysis

A total of 301 *E. asburiae* genomes were collected from the NCBI GenBank database till August 12, 2022, and 34 *E. asburiae* isolates were classified to ST252. The phylogenetic tree generated by cgSNP strategy was performed using Snippy v4.6.0 (https://github.com/tseemann/snippy). Strain 5549 was used as a reference genome for other ST252 *E. asburiae* strains. The SNP distance between two strains was calculated by snp-dist v0.8.2 (https://github.com/tseemann/snp-dists). Fasttree v2.1.11 [35] infers an approximately-maximum-likelihood phylogenetic tree from these non-recombinant SNPs. Initial clusters were identified from the phylogenetic tree by running RhierBAPS [36]. Visualization of the phylogenetic tree was created by the Interactive Tree of Life (iTOL v6) [37]. The amino acid sequence similarities among various MCR variants were compared using Clustal Omega (http://www.clustal.org/omega/).

2.10. Nucleotide sequence accession numbers

The completed sequenced whole genomes of strain 5549 (chromosome and plasmids) have been deposited in the NCBI GenBank database under accession numbers CP093154 - CP093156.

3. Results

3.1. Antimicrobial susceptibility testing

E. asburiae strain 5549 was resistant to most antimicrobial agents tested, including ceftazidime (CAZ), cefotaxime (CTX), ciprofloxacin (CIP), cefepime (FEP), cefoxitin (FOX), levofloxacin (LVX), meropenem (MEM), imipenem (IPM), trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), ampicillin-sulbactam (SAM), ceftazidime-avibactam (CAS), and a high-level resistance to colistin (MIC $>$ 128 mg/L). However, it remained susceptible to aztreonam (ATM), amikacin (AMK), gentamicin (GEN), tigecycline (TGC) (Table S2).

3.2. Identification and transferability of antibiotic resistance genes

S1-PFGE and Southern hybridization confirmed that *mcr-10* and bla_{NDM-1} were located on ~138.9–173.4 kb plasmid p5549_mcr-10 and ~54.7–78.2 kb plasmid p5549_NDM-1, respectively (Fig. S1). Transconjugation experiments showed that p5549_NDM-1 could be successfully transferred to *E. coli* J53, at a transconjugation frequency of 1.12×10^{-2} per recipient cell. Unfortunately, p5549_mcr-10 failed to produce transconjugants after repeated transconjugation experiments several times.

3.3. Functional assessment of mcr-10

To determine whether *mcr-10* mediate colistin resistance, it was cloned into pCR2.1-Hyg vector and introduced into *E. coli* DH5α strain (MIC = 0.06 mg/L). No statistical significance was observed in the expression levels of *mcr-10* genes in strain 5549 and DH5α-mcr-10 (Fig. S2). The transconjugant was also susceptible to colistin (MIC = 0.125 mg/L). Based on the amino acid similarity matrix of MCR variants, it was observed that MCR-3, MCR-7, and MCR-9 exhibited a close relationship with MCR-10, sharing amino acid identities of 62.34%, 58.85% and 82.93%, respectively. Furthermore, MCR-1, MCR-2 and MCR-6 shared a high degree of sequence similarity. Specifically, MCR-1 exhibited an amino acid sequence identity of 82.95% and 81.41% to MCR-2 and MCR-6, respectively (Fig. S3).

3.4. The relative expression levels of two-component system and MgrB

It is speculated that the high-level resistance to colistin of strain 5549 might be chromosome mediate. Nevertheless, our investigation revealed no significant differences in the mRNA expression levels of the PhoP, PhoQ, PmrA, PmrB and MgrB between *E. asburiae* ATCC 35953 (NCBI GenBank accession no. CP011863) and strain 5549 (Fig. S4).

3.5. Lipid A palmitoylation

Mass spectrum analysis was performed on both Strain 5549 and *E. asburiae* ATCC 35953 to validate the lipid A modification in colistin-resistant strain. A previous study reported [38] that the peak at *m/z* 1825 corresponds to the fundamental structure of lipid A in *Enterobacter* species. Furthermore, the peak at *m/z* 1797 corresponds to a two-carbon reduction at the C'− 2 acyl-oxo-acyl chain for the peak of *m/z* 1825. We identified two predominant peaks at *m/z* 1797, *m/z* 1825 in *E. asburiae* ATCC 35953 and three predominant peaks at *m/z* 1797, *m/z* 1825, *m/z* 2063 in strain 5549 (Fig. 1). This mass shift, *m/z* 1825–2063 (C_{16}, Δ*m/z* 238) represents the addition of palmitate to the lipid A phosphate group. Lipid A palmitoylation was detected in the colistin-resistant strain 5549, in contrast to the colistin-susceptible reference strain.

3.6. Phylogenetic analysis of global ST252 E. asburiae

MLST analysis showed that strain 5549 belongs to ST252, which

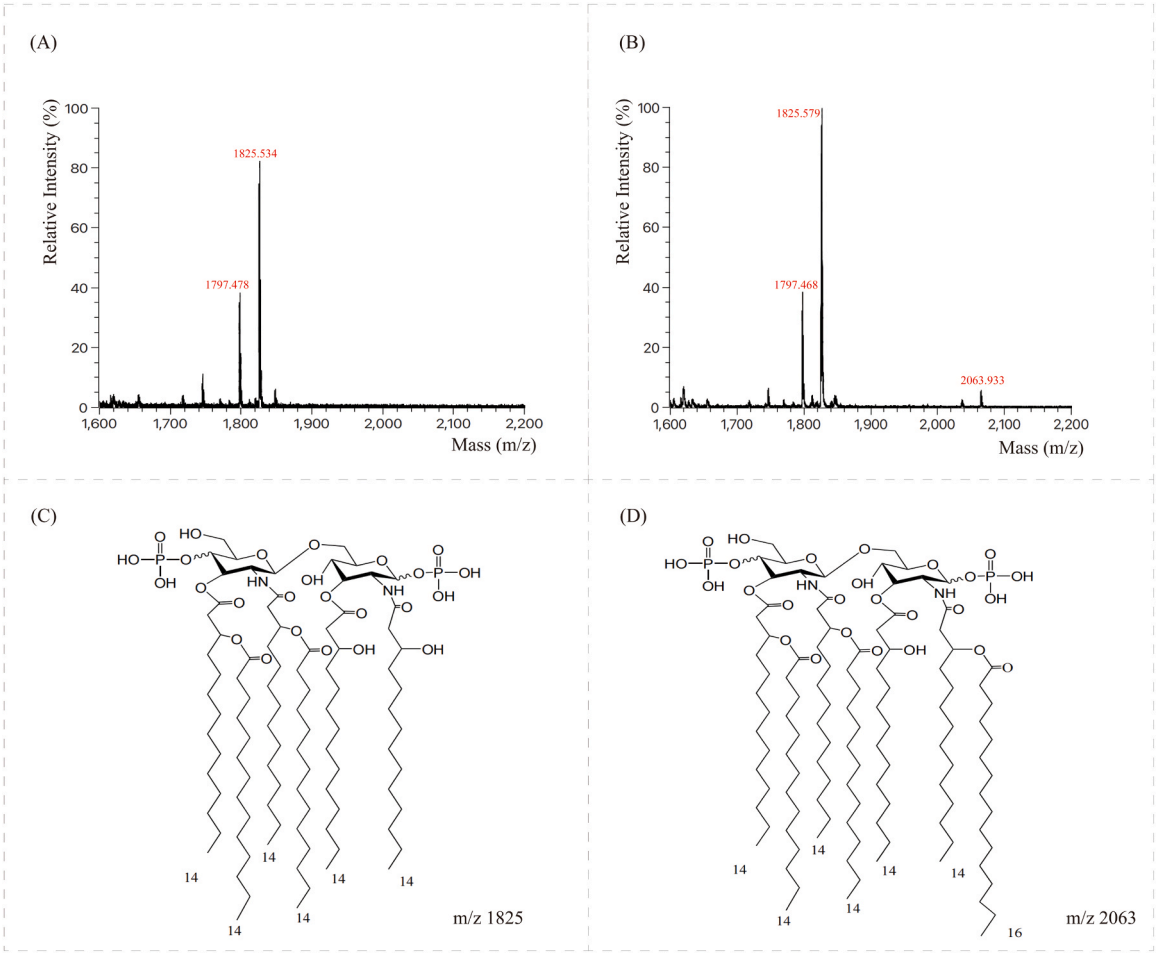

Fig. 1. Mass spectra of lipid A in colistin-susceptible and colistin-resistant *E. asburiae* strains. (A) MS analysis of the colistin-susceptible strain ATCC 35953. Peaks at *m/z* 1797, *m/z* 1825 represent the base lipid A structure along with a two-carbon reduction of an acyl chain and the base lipid A structure, respectively. (B) MS analysis of the colistin-resistant strain 5549. Mass shift, *m/z* 1825–2063 (Δ*m/z* 238) represents the addition of palmitate to lipid A. (C) Lipid A chemical structure for base peak in *Enterobacter* spp. (D) Palmitoylation of the C-1acyl-oxo-acyl chain in the chemical structure of lipid A.

accounted for 11.2% (34/302) of all *E. asburiae* retrieved from NCBI GenBank database. The global *E. asburiae* isolates were separated into 9 diverse clusters, and ST252 isolates were classified to cluster III. *E. asburiae* isolates from China were dominantly divided into I cluster (27/64, 42.1%), however, isolates from the US were evenly distributed into I, II and III clusters (Fig. 2). The detailed information of these isolates was described in Table S2. Core-genome SNP phylogenetic comparison of strain 5549 in this study with other ST252 *E. asburiae* strains (n = 34) from the NCBI GenBank database indicated that the closest relative of strain 5549 was AR2284-yvys (differing by 304 SNPs), which was isolated from the sputum of a patient with pneumonia in Hangzhou, China (Fig. 3). ST252 *E. asburiae* is sporadically distributed around the world, but most of them were isolated from patients in the United States. Among them, only four *mcr-9* and bla_{ACT}, bla_{CTX}, bla_{OXA}, bla_{TEM}, bla_{KPC} gene co-carrying isolates (AR2284-yvys, 16876-yvys, F19211027, and strain39) were identified.

3.7. Genetic characteristics of mcr-10-bearing plasmid and bla_{NDM-1}-bearing plasmid

Analysis of whole genome sequencing data revealed that strain 5549 contained a 4.80 Mb sized chromosome and two plasmids, p5549_mcr-10 and p5549_NDM-1, with the sizes of 159417 bp and 63489 bp, respectively. The chromosome harbored multiple antimicrobial resistance genes *qnrE1*, *oqxB9*, *oqxA10*, and *fosA*, which contribute to the resistance against quinolone, phenicol, and fosfomycin, respectively. Several chromosomal virulence genes *csgG*, *entA*, and *ompA*, which are responsible for the establishment of the infection. Curli fiber expression gene *csgG* encodes proteins required for biofilm formation. *entA* acts as a siderophore, facilitating bacterial invasion and promoting host infection. As a membrane protein, OmpA plays a crucial role in facilitating the adhesion and internalization processes within host epithelial cells. p5549_mcr-10 plasmid belonged to IncFIB/IncFII types and the replicon type of p5549_NDM-1 was IncN. Unlike the *mcr-10*-bearing plasmid did not carry other antimicrobial resistance genes, the bla_{NDM-1}-bearing plasmid had multiple resistance genes, including bla_{NDM-1}, *dfrA14*, *qnrS1*, and ble_{MBL}, which mediated resistance to β-lactams, trimethoprim, quinolone, and bleomycin.

BLAST performing on NCBI nucleotide database revealed that the most similar backbone to p5549_mcr-10 was pAR2284_2 (accession number CP083832, 99.99% nucleotide identity, 70% coverage), but pAR2284_2 did not carry any *mcr*-like genes. IncFIA/IncFII-type plasmid pSTW0522–51–1 (accession number CP056561) shared 56% coverage and 99.92% nucleotide identity with p5549_mcr-10 (Fig. 4A). The type-IV secretion system (T4SS) encoding in p5549_mcr-10 plasmid was interrupted by IS*3*, and no gene encoded the origin site of DNA transfer (oriT) (Fig. 4A). The best match of p5549_NDM-1 was the IncN-type plasmid pNDM_cf7308 (accession number CP092465.1), with 99.97% identity and 98% coverage (Fig. 4B). The bleomycin resistance protein gene ble_{MBL} located downstream of bla_{NDM-1} gene, the genetic context of bla_{NDM-1} is conserved because the IS*30*-bla_{NDM-1}-ble_{MBL}-*cutA*-*groES*-*grol* structure was all identified in different plasmids. Compared with the

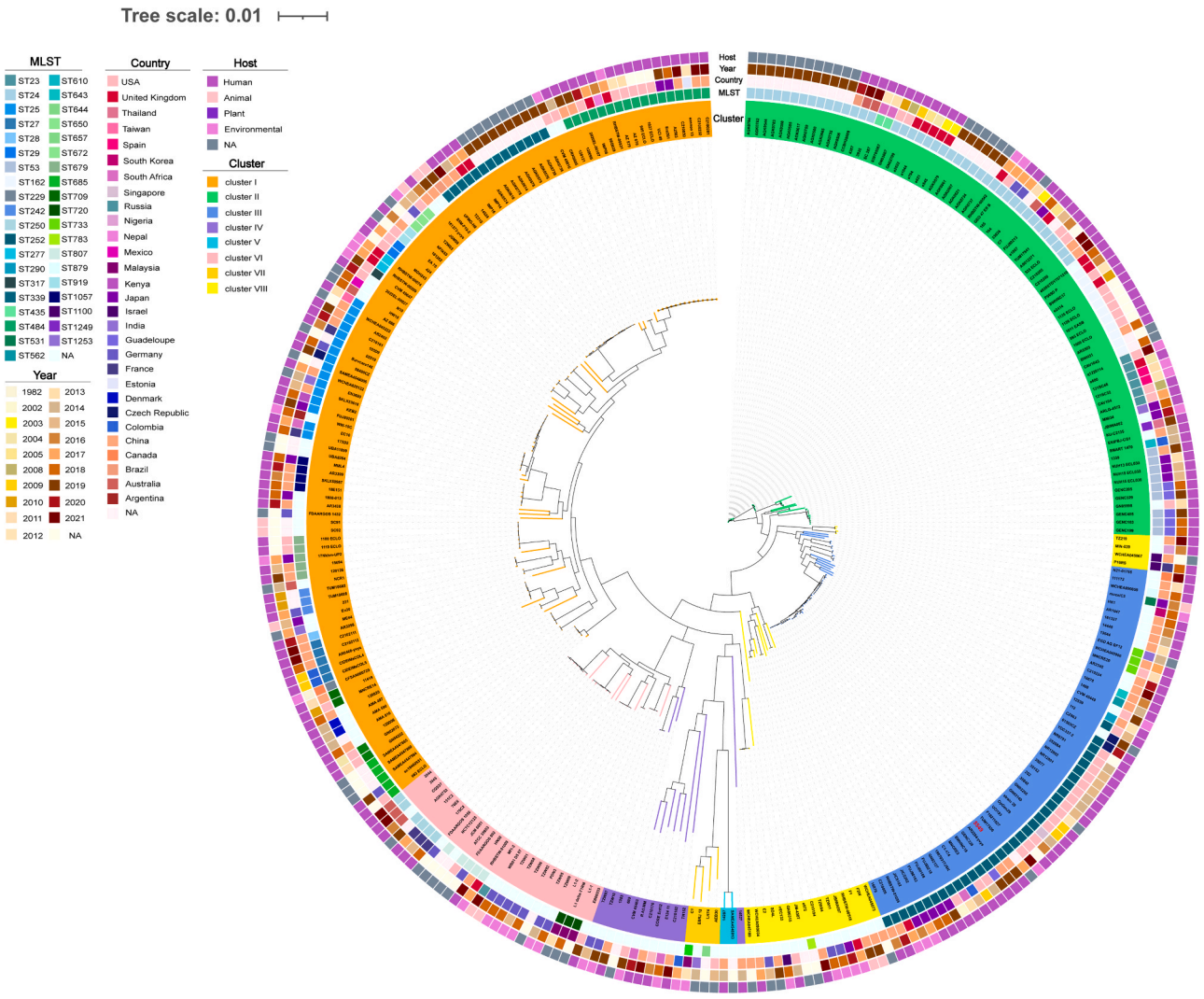

Fig. 2. Phylogenetic analysis of all 301 *E. asburiae* from NCBI GenBank database, the strains identifier (ID), geographical location, separation time, hosts are shown. Diverse clusters are marked in different colors.

other similar plasmids, insertion of an additional IS*Ec33* sequence between IS*30* and Tn*3* located downstream of bla_{NDM-1} gene was found in p5549_NDM (Fig. 5B).

To further explore the transconjugation mechanism of *mcr-10*, we selected five *mcr-10*-bearing plasmids belonged to IncFIB/IncFII types with identity> 99% in NCBI GenBank. The tyrosine-type recombinase gene *xerC* was located upstream of the *mcr-10* in all the *mcr-10*-bearing plasmids we analyzed. The high conservation of *xerC-mcr-10* in strain 5549 is also in line with the previously studies [21]. Various insertion sequences were found in the upstream or/and downstream regions of *mcr-10*, while the genetic context of *mcr-10* in the p5549_mcr-10 was different from others without any insertion sequences. The genes encoding conjugative transfer relaxase/helicase TraI and type IV conjugative transfer system coupling protein TraD were located at downstream of *mcr-10* and the upstream structure of *xerC* is partly similar to that of pEk72–1, pSTW05222–51–1 and p161373–1 (Fig. 5A).

4. Discussion

E. asburiae was originally isolated from a patient in the United States in 1986, which showed resistance to amoxicillin, cefazolin and cefoxitin, but still susceptible to colistin [39]. It has been isolated from different sources, including urine, stools, wounds, but mainly in blood cultures. Previous studies reported that the most prevalent colistin-resistant species found in ECC were *E. asburiae*, which might possess intrinsic resistance determinants [13]. The first isolation of a carbapenem-resistant *E. asburiae* was recovered from the US rivers carrying plasmid mediated bla_{IMI-2}, and the number of carbapenem-resistant *E. asburiae* has increased recently [40]. In our study, all *E. asburiae* strains retrieved from NCBI GenBank database comprised of 39 different sequence types (STs), and the dominant one was ST24 (41/302, 13.5%), followed by ST252 (34/302, 11.2%), and ST484 (17/302, 5.6%). The *E. asburiae* ST252 strains were isolated from various geographical locations, with most of them circulating across the United States from various sources. Previous studies have reported *mcr-10* could be detected from different species, such as *K. pneumoniae*, *E. coli*, *Cronobacter sakazakii* [41–43], and ECC were regarded as the predominant species. The *mcr-10* gene was mainly located in *E. hormaechei*, *E. roggenkampii* and *E. kobei* among ECC, and the prevalence of *mcr-10* in *E. asburiae* is still low [23]. Since *mcr-10* was first identified from a colistin-susceptible *E. roggenkampii* strain, *mcr-10*-bearing self-transmissible plasmids in *Enterobacter* spp. from animal and hospital sewage water have been reported consecutively. Notably, under laboratory conditions, *mcr-10*-carrying plasmids could be transferred successfully, but the transconjugants did not exhibit a colistin-resistant phenotype. These findings indicate a silent dissemination scenario of *mcr-10* among various gram-negative bacteria, particularly in ECC [20,21].

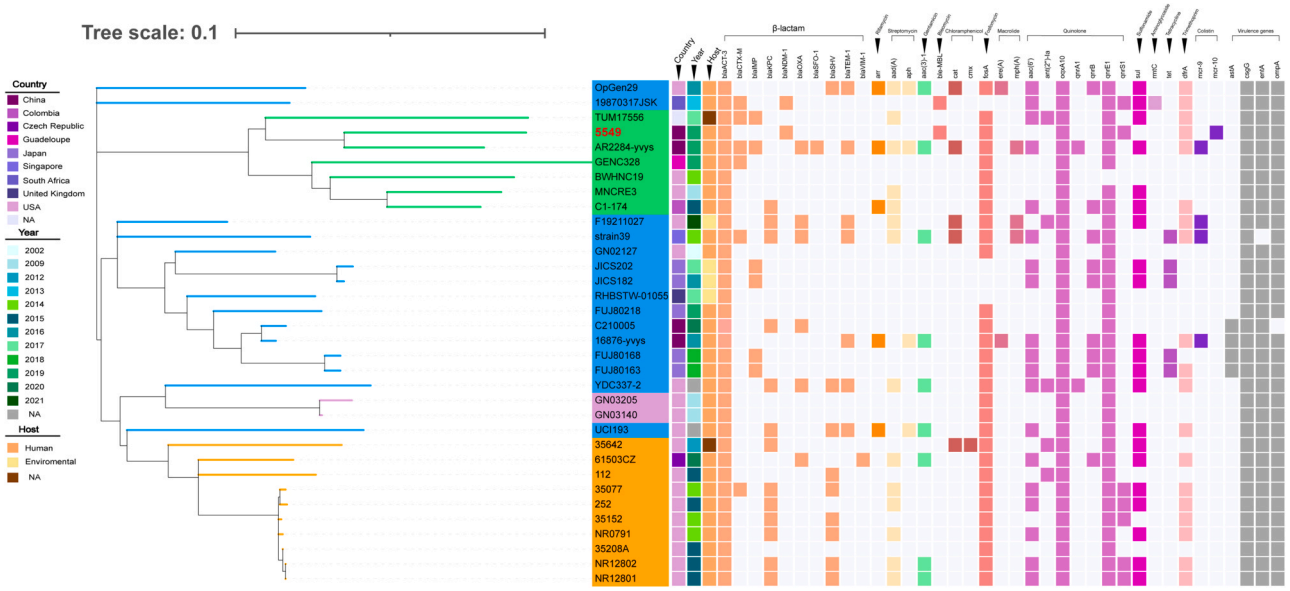

Fig. 3. Phylogenetic analysis of *E. asburiae* strain 5549 and other ST252 *E. asburiae* strains acquired from NCBI GenBank database. The strains identifier (ID), geographical location, separation time, hosts, antimicrobial resistance genes and virulence genes are shown. The cell in different colors represents the presence of the gene, while the blank cell represents the absence of the gene. Diverse clusters are marked in different colors.

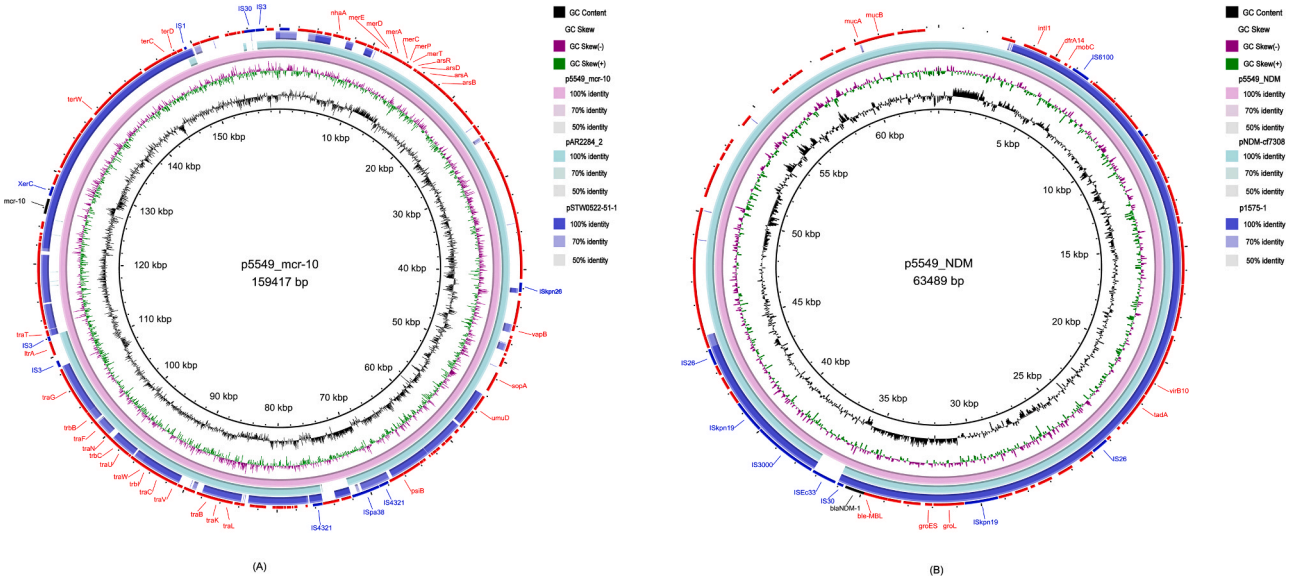

Fig. 4. Genetic comparison *mcr-10*-bearing plasmid and bla_{NDM-1}-bearing plasmid from strain 5549 with similar plasmids in the NCBI database, respectively. (A) Alignment of two similar plasmids pAR2284_2 (CP083832) and pSTW0522–51–1 (AP022432). (B) Alignment of two similar plasmids pNDM-cf7308 (CP092465) and p1575–1 (CP068288), black region indicates *mcr-10* gene and bla_{NDM-1} gene.

The emergence of multidrug-resistant ECC strains has reduced the susceptibility of last-resort antibiotics, especially carbapenems and colistin. Of more concern, the carbapenemase gene and *mcr* gene co-carrying ECC strains had a high prevalence in clinical isolates, therefore, understanding the mechanisms of transmission and multidrug resistance is critical to control the global spread of carbapenem and colistin resistant ECC. To date, the *mcr-9* gene has been associated with the co-occurrence of carbapenemase genes bla_{VIM}, bla_{IMP}, bla_{KPC}, and bla_{NDM-1} [10,24,44,45], however, case reports about co-existence of *mcr-10* and carbapenemase genes ECC are still rare. A study has reported that the bla_{ACT} gene was most prevalent among the ECC and its variants (bla_{ACT-2}, bla_{ACT-3}) were only present in *E. asburiae* [46]. In our findings, the bla_{ACT-3} genes were identified in all ST252 *E. asburiae*, indicating that it serves as a reservoir for bla_{ACT-3} genes. Besides, bla_{KPC} was identified accounting for 38.2% (13/34) in ST252 *E. asburiae*, followed by bla_{IMP} (6/34, 17.6%) and bla_{NDM-1} (2/34, 5.8%).

A previous study revealed that a high prevalence of *mcr-9* and carbapenemase genes co-existing in ECC from 2000 to 2018 [18]. Different from a highly conserved plasmid type (IncHI2/2 A) was identified in *mcr-9*-carrying plasmids, both transferable and nontransferable *mcr-10*-carrying plasmids were found to belong to various plasmid types so far, such as IncFII, IncFIA, IncFIB, IncFIB/IncFII. The silent dissemination of *mcr-10* on diverse Inc-type of plasmids improved the ability of *mcr-10*-positive bacteria to survive colistin selection [47]. *mcr-10* genes located downstream of *xerC* genes in all the *mcr-10*-bearing plasmids we analyzed, and the structure of *xerC-mcr-10* was quite conservated. It has been shown that the carbapenemase genes (bla_{IMI}, bla_{NMC-A}) were integrated into the ECC by *xerC*-mediated site-specific

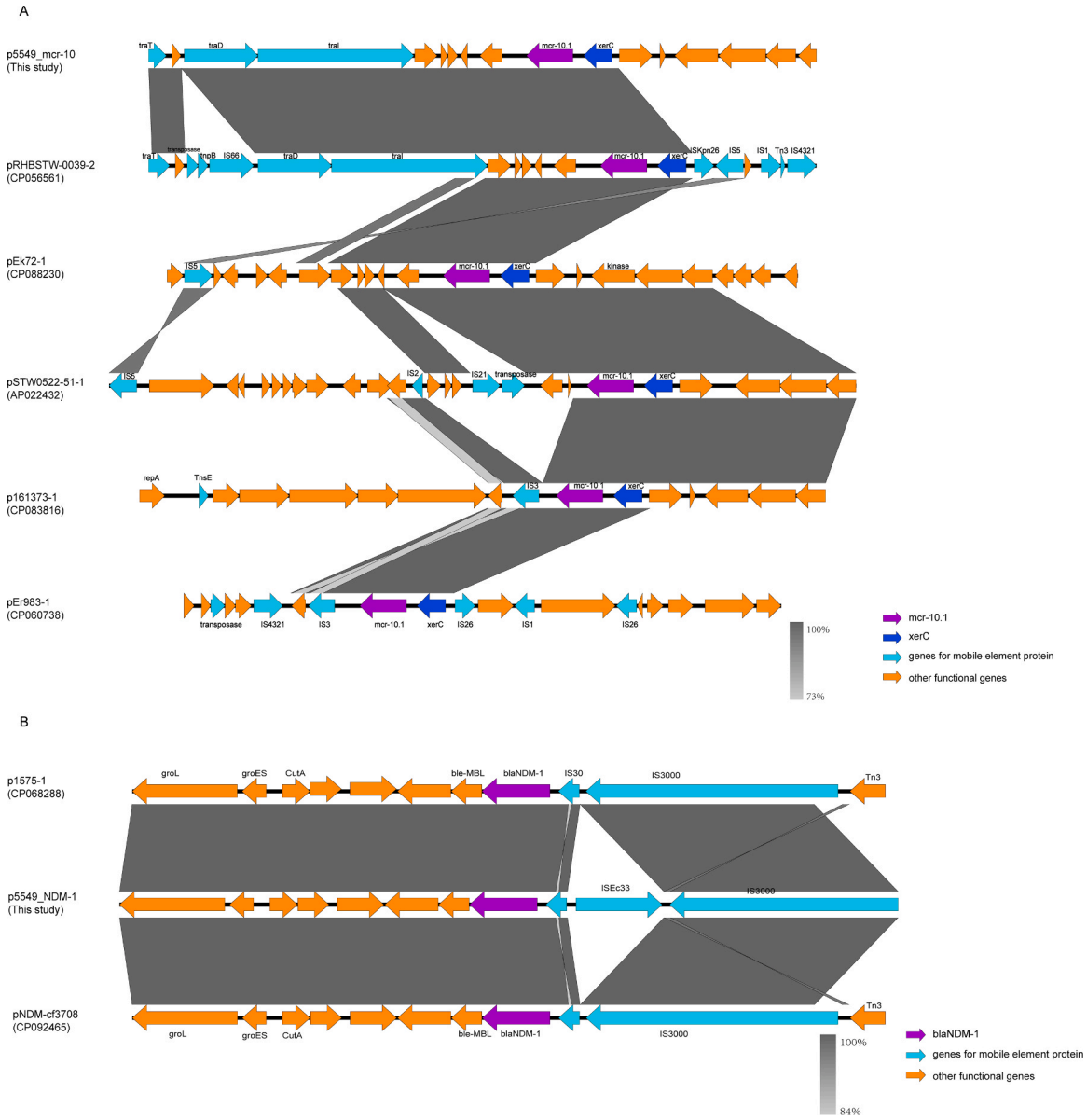

Fig. 5. Genetic contexts of *mcr-10* and bla_{NDM-1} and comparison of the homologous regions shared by p5549_mcr-10 (A), p5549_NDM (B) and other *mcr-10* and bla_{NDM-1}-bearing plasmids deposited in the GenBank database. Different genes are shown in different color.

recombination [48]. Therefore, the *xerC*-type tyrosine recombinase may also contribute to *mcr-10* mobilization. The *mcr-10* gene was mainly detected on self-transmissible plasmids in *E. roggenkampii* [20,21]. Moreover, a potential site-specific recombination mechanism through integrase-mediated *mcr-10* transmission in *mcr-10* positive *Cronobacter sakazakii* was confirmed [43]. In our study, *mcr-10* was cloned into *E. coli* DH5α without increase the colistin MIC, which is accordant with previous studies that *mcr-10* consisted in colistin-susceptible strains [11, 49]. This finding illustrates that *mcr-10* did not contribute to the colistin resistance in strain 5549. MCR-3, MCR-7, and MCR-9 demonstrated close relationships with MCR-10 among the analyzed MCR variants. MCR-10 shared the highest amino acid identity of 82.93% with MCR-9. Previous studies have reported that ECC strains carrying both *mcr-10* and *mcr-9* remained susceptible to colistin [44]. Nevertheless, *mcr-3* and *mcr-7* confer a low level of resistance to colistin [50,51]. MCR-1, exhibiting notable difference from MCR-10 at the amino acid level, has been proven to confer a moderate colistin resistance since its initial report [6]. The additional contribution of *mcr* gene in colistin resistance deserved our attention, such as defense system against the host immune system, natural antimicrobial peptide, bacteriophage predation, or even enhancing the virulence potential of bacteria [52]. Moreover, we should pay attention to the high-level of colistin resistance. According to a previous report, *E. asburiae* accounted for 40% among MIC of colistin $>$ 64 mg/L ECC isolates, suspecting that colistin resistance was *E. asburiae* species-dependent [13]. Besides, Guérin et al. revealed that the colistin hetero-resistance in the ECC appeared to be cluster-dependent [53]. The overexpression of *acrAB-tolC* efflux pump regulated by *soxRS* was recently reported in colistin hetero-resistance *E. cloacae* and *E. asburiae* isolates [54].

Many studies have demonstrated that resistance to colistin in Enterobacteriaceae was mediated mainly by the two-component systems PmrA/B, PhoP/Q and its negative feedback regulatory protein MgrB [55,56]. As for colistin hetero-resistant ECC, a small transmembrane protein Ecr was found through PhoP/Q two-component system to mediate colistin resistance [57], and several cases reported that mutations of PhoP/Q and its inhibitor MgrB might be related to colistin resistance, but PmrA/B does not [12]. In our study, no statistically significant differences were observed in the expression levels of TCSs

(PhoP/PhoQ and PmrA/PmrB) and MgrB between colistin-susceptible strain and colistin-resistant strain ($P > 0.05$), indicating that it was an alternative molecular mechanism of high-level colistin resistance in *E. asburiae*. Moreover, a previous study has indicated that there is co-upregulation of *mcr-10* and *phoP/phoQ* at the transcriptional level when exposed to colistin, suggesting that *mcr-10* might play a functional role in resisting colistin pressure [21]. However, in the strain 5549, which exhibits high-level resistance to colistin, the expression levels of *mcr-10* and other genes associated with colistin resistance did not demonstrate an upward trend even under strong selective pressure of increasing colistin concentrations from 64 mg/L to 1024 mg/L (data not shown). Consequently, the presence of colistin failed to induce the expression of *mcr-10* in our strain.

In ECC, colistin resistance has been attributed to LPS modifications. The L-Ara4N production and transfer to lipid A are mediated by the active *arn* operon [57]. In addition, *mcr* genes are PEtN transfer enzymes for mediating transfer of PEtN to lipid A [58]. This study observed the mass shift m/z 1825–2063 (C16, $\Delta m/z$ 238) in strain 5549, corresponding to the lipid A palmitoylation. This modification of lipid A has been described as alternative route to colistin resistance in *K. pneumoniae* [59]. Consistent with a previous study [13], lipid A palmitoylation has also been identified in colistin-resistant ECC. Nevertheless, further investigations are needed to elucidate the precise role and implications of lipid A palmitoylation in colistin resistant ECC.

In conclusion, our study reports the first multidrug-resistant ST252 *E. asburiae* isolate co-carrying *mcr-10* and bla_{NDM-1} genes in China. The presence of lipid A palmitoylation contributes to the high-level of colistin resistance in *E. asburiae*. It is imperative to further monitor and control the transmission of colistin and carbapenem resistance in *E. asburiae*.

Funding

This work was supported by the National Natural Science Foundation of China (82202565, 81871696), the Zhejiang Provincial Natural Science Foundation of China (LR23H200001, LY21H190001) and the Zhejiang Provincial Medical and Health Science and Technology Plan (2020RC066, 2021RC007).

CRediT authorship contribution statement

Xinyang Li: Methodology, Software, Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. **Tian Jiang:** Methodology, Data curation, Formal analysis, Investigation, Writing – review & editing. **Chenghao Wu:** Data curation, Formal analysis, Investigation, Validation. **Yingying Kong:** Data curation, Formal analysis, Investigation. **Yilei Ma:** Formal analysis, Investigation. **Jianyong Wu:** Formal analysis, Investigation. **Xinyou Xie:** Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Jun Zhang:** Project administration, Supervision, Writing – original draft, Writing – review & editing. **Zhi Ruan:** Conceptualization, Funding acquisition, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.08.004.

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