Infection and Drug Resistance

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ORIGINAL RESEARCH

Genomic and Phenotypic Characterization of a Colistin-Resistant *Escherichia coli* Isolate Co-Harboring bla_{NDM-5}, bla_{OXA-1}, and $bla_{CTX-M-55}$ Isolated from Urine

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Background: Colistin is one of the few options for treating carbapenem-resistant Enterobacterales (CREs). There is little available information about the epidemic status of colistin-resistant CREs in Southern Sichuan, China. This study mainly investigated the genomic and phenotypic characteristics of an extensively drug resistant *E. coli* LZ00114 isolated from Luzhou, China.

Materials and Methods: In 2020, LZ00114 was isolated from the urine of a patient with hydronephrosis and urinary tract infection in Luzhou, China. We assessed the resistance profile of LZ00114 in the presence and absence of the protonophore carbonyl cyano-m-chlorophenylhydrazine (CCCP) and 1-(1-naphthylmethyl)-piperazine (NMP) by antimicrobial susceptibility testing. The growth kinetics, motility, and pathogenicity of LZ00114 were determined to evaluate its microbial characteristics. In combination with whole genome sequencing (WGS) and real-time reverse transcription PCR (RT-PCR), we comprehensively analyzed the resistance mechanisms of LZ00114.

Results: LZ00114 was resistant to various antimicrobial agents, including meropenem, tetracycline, ciprofloxacin, gentamicin, fosfomycin, and polymyxin B. Notably, CCCP reversed the resistance of LZ00114 to polymyxin B. LZ00114 displayed high pathogenicity in the infection model ($P<0.01$) compared with the Lab-WT strain, and its growth rate and motility were not significantly different from the Lab-WT strain. WGS and conjugation revealed that LZ00114 belonged to ST410 and carried a bla_{NDM-5}-harboring self-transmissible IncX3 plasmid and a multi-replicon IncFII/FIA/FIB plasmid carrying bla_{OXA-1}-$bla_{CTX-M-55}$-*tet(B)-aac(6')-Ib-cr-dfrA17-sul1-fosA3*. Comparative genomics revealed genetic relatedness between LZ00114 and strains isolated from other regions. Furthermore, there were point mutations in *pmrA* (S29G, G144S), *pmrB* (D283G, Y358N), *marR* (G103S, Y137H), *emrA* (I219V), and *emrD* (G323D) of LZ00114. RT-PCR confirmed the overexpression of efflux pumps and PmrABC in LZ00114.

Conclusion: This study provides valuable information for the surveillance of antimicrobial resistance and a theoretical basis for the prevention and control of colistin-resistant *E. coli*. There is still a need to be vigilant about the clone spread of the high-risk clone group *E. coli* ST410.

Keywords: *E. coli*, antimicrobial resistance, whole genome sequencing, colistin, carbonyl cyanide m-chlorophenylhydrazine, CCCP, ST410

Introduction

Escherichia coli is a common Gram-negative opportunistic pathogen that causes invasive host infections through virulence factors such as flagella, toxin secretion, and adhesins. According to the source of the infection, pathogenic *E. coli* can be classified as intestinal (diarrheagenic) and extraintestinal (ExPEC). Uropathogenic *E. coli* (UPEC) is the

Received: 31 December 2021
Accepted: 24 February 2022
Published: 29 March 2022

most common lineage of ExPEC. In hospitals, the likelihood of opportunistic infections is high in immunocompromised patients as well as those needing catheters.[1] These pathogens adhere to the uroepithelium through the synergistic actions of various virulence factors and cause ascending infections of the bladder, kidneys, and blood by regulating the movement of bacterial pili.[2,3] However, the emergence of antimicrobial resistance makes treating these bacterial infections a thorny problem.[4] ST131 was previously considered the central lineage of antimicrobial resistance transmission in extraintestinal *E. coli*, with ST167 and ST410 gradually emerging as new high-risk clones.[5–7] For these strains, carbapenemase production is the primary mechanism of carbapenem resistance in Enterobacterales. The worldwide epidemic of bla_{NDM}-producing Enterobacterales has caused widespread concern. Based on NDM-1, several NDM variants with higher levels of carbapenem resistance have been identified.[8] IncX and IncF plasmids are usually the primary mediators of bla_{NDM} dissemination. They can carry various acquired antimicrobial resistance genes to promote the formation of multidrug-resistant bacteria and expand the host range. Although fitness cost usually accompanies acquired resistance, bacteria can reduce this effect through additional chromosomal mutation, epistatic effects, and environmental factors.[9] Growth, motility, and pathogenicity are often important factors in evaluating the resistance cost of resistant bacteria. Therefore, it is necessary to study the microbial characteristics of these pathogens to assess the alterations in their fitness.

Avibactam, a new β-lactamase inhibitor, does not inhibit the activity of metallo-beta-lactamases (MBLs), and colistin is one of the primary drugs for treating MBLs-producing MDR and XDR resistant Enterobacterales. Therefore, once the MBL-producing CRE develops colistin resistance, it will further limit antimicrobial therapy. In *E. coli*, colistin resistance is usually associated with the two-component system, namely, the PmrAB, PhoPQ-mediated lipopolysaccharide (LPS) modification, and the acquisition of exogenous *mcr* genes.[10] For two-component mediated colistin resistance, other factors besides colistin can also select mutations, that is, colistin-resistant strains can be isolated from patients without colistin treatment.[10] Furthermore, the efflux pump was associated with the formation of heteroresistance to colistin.[11] In recent years, it has been found that the efflux pump inhibitor CCCP can reverse the colistin resistance of bacteria, but the exact mechanism needs to be further clarified.[12] Therefore, more valuable information is needed to elucidate the colistin resistance mechanisms in Enterobacterales. In our study, we described the isolation of a colistin-resistant extensively drug-resistant *E. coli* isolate LZ00114 from urine, which to our knowledge is also the first report of colistin-resistant *E. coli* isolated from Southern Sichuan, China. In addition, we focus on its molecular genetics and microbial characteristics. The results of this study provide a theoretical basis for the prevention and control of colistin-resistant *E. coli* worldwide.

Materials and Methods

Bacterial Isolate

In April 2020, LZ00114 was isolated from a female patient with hydronephrosis and urinary tract infection in the Affiliated Hospital of Southwest Medical University (Luzhou, China). This isolate was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) as *E. coli*. At that time, the clinical microbiology laboratory reported that LZ00114 was resistant to beta-lactams, carbapenems, fluoroquinolones, tetracyclines, chloramphenicol, and gentamicin. The patient was treated with tigecycline and showed improved symptoms of the infection. During hospitalization, the patient was not treated with colistin. To explore whether LZ00114 is also resistant to colistin and fosfomycin and to define its resistance mechanisms, as well as the microbial characteristics of uropathogenic *E. coli*, we performed a follow-up study.

Antimicrobial Susceptibility Testing

We tested the minimum inhibitory concentration (MIC) breakpoints of 14 antimicrobial agents according to the definition of extensive drug resistance.[13] The MIC breakpoints of ampicillin, aztreonam, cefepime, ciprofloxacin, tetracycline, meropenem, imipenem, chloramphenicol, and tigecycline were determined using the MicroScan WalkAway System (Siemens, Germany). The microbroth dilution method based on the 96-well plate was used to determine the MIC value of polymyxin B. The MIC of fosfomycin was measured using the agar dilution method. The antimicrobial susceptibility

results were interpreted according to CLSI 2020-M100.[14] The MIC of tigecycline was interpreted using the guidelines of the European Committee on Antimicrobial Susceptibility Testing (2021, https://www.eucast.org/). The *E. coli* ATCC25922 strain served as the quality control.

Detection of Carbapenem Genes, Chromosome, and Plasmid-Mediated Colistin Resistance Genes

Total DNA was extracted from bacteria using the boiling method.[15] In brief, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}, *pmrAB, phoPQ* and *pmrD* genes were detected by polymerase chain reactions (PCR). All PCR primers used in this study were listed in Table S1. Positively amplified products were sequenced by the Sanger sequencing method and compared with the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).

Efflux Pump Inhibition Assay

To assess the role played by efflux pumps in antimicrobial resistance, we evaluated the changes in MICs in the presence of efflux pump inhibitors, namely, CCCP (Sigma, USA) and NMP (Alfa Aesar, CN). The final concentrations of CCCP and NMP were 10 mg/L and 100 mg/L, respectively. A 4-fold or more reduction in the MICs after adding the efflux pump inhibitors was interpreted as a high expression of the efflux pump, and an 8-fold or more reduction in the MIC of colistin after adding CCCP was interpreted as colistin resistance reversal.[16,17]

Galleria mellonella Infection Model

To assess pathogenicity, the virulence of bacteria was modeled as previously described using *Galleria mellonella* larvae, with minor modifications.[18] The concentrations of the bacterial suspensions were adjusted to 1×10^7 CFU/mL. Hamilton precision syringes were used to inject 10 μL of the bacterial suspension (the actual inoculation volume was 10^5 CFU) into 15 healthy larvae, followed by incubation at 37°C for 72 h in the dark. The number of nonviable larvae was recorded every 24 h. The Lab-WT *E. coli* ATCC25922 strain served as the negative control. All assays were conducted in triplicate.

Biofilm Formation Capacity, Growth Kinetics, and Motility Assays

The biofilm formation capacity of the isolate was measured by staining with 0.1% crystal violet as previously described with minor modifications.[19] The plates were incubated at 37°C for 24 h, washed, fixed, stained, and solubilized with 95% ethanol. Subsequently, the absorbance was measured at 570 nm with a microplate reader (PerkinElmer, USA). The *A. baumannii* ATCC19606 and sterile LB broth served as positive and negative controls, respectively. The data were expressed as mean ± SD. All assays were conducted in triplicate.

For growth kinetics, briefly, 10 μL of 0.5 McFarland turbidity standard suspension was added to each well of a 96-well plate, followed by 190 μL of LB broth per well. Three replicate wells were set up for each strain. The absorbance was measured at 600 nm at 2 h intervals at 37°C.[20] Motility tests were performed as previously described with minor modifications.[21] In brief, 1.5 μL of 0.5 McFarland turbidity standard suspension was added to 0.25% tryptone agar plates. The plates were incubated for 16 h at 35°C. All assays were conducted in triplicate. The experimental results were compared with Lab-WT (Wild Type) *E. coli* ATCC25922.

Conjugation

E. coli J53 was used as the recipient, and LZ00114 *E. coli* was used as the donor. The two strains were added to antibiotic-free LB broth at a ratio of 2:1 and incubated for 24 h. MH plates containing sodium azide (180 μg/mL) and meropenem (4 μg/mL) were used to screen the transconjugants.

Whole Genome Sequencing and Analysis

Total genomic DNA was extracted using a magnetic-bead–based kit (Qiagen, Germany). The purified DNA was used to construct the library according to the NEBNext Ultra DNA Library preparation kits and sequenced using Illumina NovaSeq 6000 PE150 and Oxford nanopore platforms. Read sequences were de novo assembled using continuous long

reads following Canu software (version 1.7). Prokka software (version 1.10) was engaged to predict the coding genes, tRNAs, and rRNAs in the assembled genome. The resistance genes, serotypes, and plasmid types were analyzed by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/), with the threshold for identity using the default settings for web pages. Virulence genes were identified by VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) and virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/). The BLAST (http://blast.ncbi.nlm.nih.gov), OAT software (v 0.931), and BRIG (BLAST Ring Image Generator) v0.95 software were used for comparative genomic analysis.[22] The analysis of average nucleotide identity (ANI) used chromosome sequences of *E. coli* ST410 strains (WCHEC025943, PT109, and AMA1167) previously deposited into the NCBI database.[23–25]A phylogenetic tree based on single nucleotide polymorphisms (SNPs) was constructed by using the CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). IslandPath-DIOMB (v 0.2) was used for genomic island prediction analysis.[26] *E. coli* str. K-12 substr. MG1655 (GenBank accession number: NC_000913.3) served as a reference strain for chromosome point mutation analysis. All genomic data (LZ00114) were stored in the NCBI database under accession numbers: CP087570, CP087571, and CP087572.

Transcriptional Expression of Efflux Pumps and PmrAB Two-Component System by Quantitative Real-Time PCR

The expression levels of resistance-nodulation-cell division (RND) efflux pump genes (*acrA, acrB*, and *acrD)*, major facilitator superfamily (MFS) efflux pump genes (*emrA, emrD*, and *emrY*), and the two-component regulatory system PmrAB were detected by RT-PCR. The selected purified colonies were inoculated in 5 mL of LB broth and cultured to the mid-exponential phase. Subsequently, total RNA was extracted using the RNA kit according to the reagent manufacturer's recommendations. The design of the RT-PCR primers is provided in Table S1, with *danE* and *gapA* serving as internal reference genes. *E. coli* ATCC25922 served as the control strain. The relative expression of the indicated genes was calculated by the $2^{-\Delta\Delta Ct}$ method. The data were expressed as mean ± SD, and statistical analysis was performed using Student's *t*-test. All assays were conducted in triplicate.

Results

General Characteristics of LZ00114

LZ00114 was resistant to β-lactams, fluoroquinolones, aminoglycosides, polymyxin B (8 μg/mL), and other common antibiotics except for tigecycline (Table 1). PCR showed that LZ00114 carried the bla_{NDM-5} gene, but the *mcr-1* gene was not identified. Using *E. coli* ATCC25922 as the Lab-WT reference strain, we tested the microbial characteristics of LZ00114. Growth kinetics showed that LZ00114 grew faster than the Lab-WT strain over 24 h, and the OD_{600} values of LZ00114 and the Lab-WT strain reached 0.6 at approximately 14 h and 16 h, respectively. As an indicator of the pathogenicity of uropathogenic *E. coli*, the motility of LZ00114 did not show significant differences compared with the Lab-WT strain. In the *G. mellonella* infection model, larvae inoculated with 10^5 CFU of LZ00114 showed a 46.7% mortality rate within 4 days, while those inoculated with 10^5 CFU of the Lab-WT strain showed a 6.7% mortality rate within 4 days (Figure 1). For biofilm formation capacity, the OD_{570} value of the negative control was 0.12±0.01, LZ00114, and *A. baumannii* ATCC19606 (positive control) exhibited moderately adherent (0.26±0.03, 2+) and strongly adhesion (0.57±0.05, 3+), respectively.

The LZ00114 genome consisted of three contigs of 4,953,723 bp containing a chromosome backbone (4,755,648 bp) and two circular plasmids. Furthermore, bla_{OXA-1} and bla_{NDM-5} were located on IncF (151,915 bp) and IncX3 (46,160 bp) plasmids, respectively. According to *silico* MLST analysis, the sequence type of LZ00114 belonged to ST410. Comparison of wzt, wzy, and flic genes by SerotypeFinder showed that the serotype was O8:H9 (identity>99.5%). Moreover, LZ00114 carried many virulence factors for iron uptake and adhesion. The Fim/fimH/fumC types, virulence factors, and acquired antimicrobial resistance genes are shown in Table 2. In particular, the chromosomal genome of LZ00114 showed highly similar ANIs (99.98%, 99.88%, and 99.99%, respectively) with *E. coli* ST410 isolate WCHEC025943 (China, GenBank accession number: CP027205.2), PT109 (Portugal, GenBank accession number: CP041031.1), and AMA1167 (Denmark, GenBank accession number: CP024801.1). Additionally, LZ00114 showed very high ANI with two *E. coli* strains from

Table 1 Antimicrobial Susceptibility and Efflux Pump Inhibition Tests of LZ00114

Antimicrobial Agents	MIC (μg/mL)		
	/	+CCCP (10 μg/mL)	+NMP (100 μg/mL)
Ceftazidime	256	256	256
Cefepime	256	256	256
Meropenem	32	8	16
Tetracycline	256	64	64
Ciprofloxacin	32	16	16
Gentamicin	32	16	16
Chloramphenicol	64	16	16
Polymyxin B	8	0.5	8
Tigecycline	0.25	–	–
Ceftazidime/Avibactam	>16/4	–	–
Aztreonam	>16	–	–
Trimethoprim-Sulfamethoxazole	>4/76	–	–
Imipenem	32	–	–
Ampicillin/Sulbactam	>32/16	–	–
Fosfomycin	>512	–	–

KBN10P04869 (Korea, GenBank accession number: CP026473.1) and RUT3575 (Spain, GenBank accession number: CP048010.1) and H10407 is an enterotoxigenic *E. coli* isolate (GenBank accession number: NC_017633.1), while the complete genome of LZ00114 and WCHEC025943 had 99.97% of ANI (Figure 2A). The phylogenetic tree showed that LZ00114 was evolutionarily closer to WCHEC025943 and AMA1167, as shown in Figure 2B.

Chromosomal Genome Analysis of LZ00114

We selected the *E. coli* K12 MG1655 as the reference strain to identify the chromosomal point mutations. There were amino acid substitutions in *gyrA* (S83L, D87N), *parE* (S458A), and *parC* (S80I) within the fluoroquinolone resistance-determining region of LZ00114. As the *mcr* genes were not identified in the genome, we speculated that chromosomal mutations mediate polymyxin B resistance. By Sanger sequencing, we observed that the PCR products of two-component regulatory genes, namely, *pmrAB* and *phoPQ* had point mutations as follows: *pmrA* (S29G, G144S) and *pmrB* (D283G, Y358N). Whole genome sequencing subsequently confirmed these results, and amino acid substitutions were found in the *eptA* (*pmrC*) gene (D348G, T413S). Furthermore, the *nfsA* gene, which encodes NADPH-dependent nitroreductase also had a point mutation (R203H). We then identified point mutations in *marR* (G103S, Y137H), *acrD* (Q656K, A740D), *ermA* (I219V), *emrD* (G323D). Subsequently, we verified the efflux pump phenotype and confirmed the presence of efflux pump overexpression by RT-PCR.

In addition, we identified a genomic island (GI) containing $bla_{\text{CMY-2}}$ with a length of 15,343 bp on the chromosome backbone of LZ00114 (4,213,994–4,229,337bp). The GC ratio of this GI was 48.87% lower than that of the other chromosomal regions. This region may have been integrated into the chromosome by mobile elements, and further analysis suggested that integration may have been accomplished by *IS600* and *IS66*-like elements at either end of the GI (Figure S2). We compared the genetic environment and found that $bla_{\text{CMY-2}}$ integrated into the chromosome lacking *blc* and *yggR* in the surrounding environment compared with plasmid-derived $bla_{\text{CMY-2}}$ (Figure S1). The $bla_{\text{CMY-2}}$ genetic environment of LZ00114 was similar to the *E. coli* strain RUT3575 (GenBank accession number: CP048010.1), with the same $bla_{\text{CMY-2}}$ located on the chromosome (Figure S1).

Characteristics of an IncX3 $bla_{\text{NDM-5}}$-Harboring Plasmid p25NDM-5 and an IncFII/FIA/FIB $bla_{\text{OXA-1}}$-Harboring Plasmid p1OXA77

$bla_{\text{NDM-5}}$ was located on a 46,160 bp IncX3 plasmid p25NDM-5 with a GC ratio of 46.7%. By BLAST-based sequence alignment using the GenBank nucleic acid sequence database, we found that two plasmids carrying $bla_{\text{NDM-5}}$ isolated

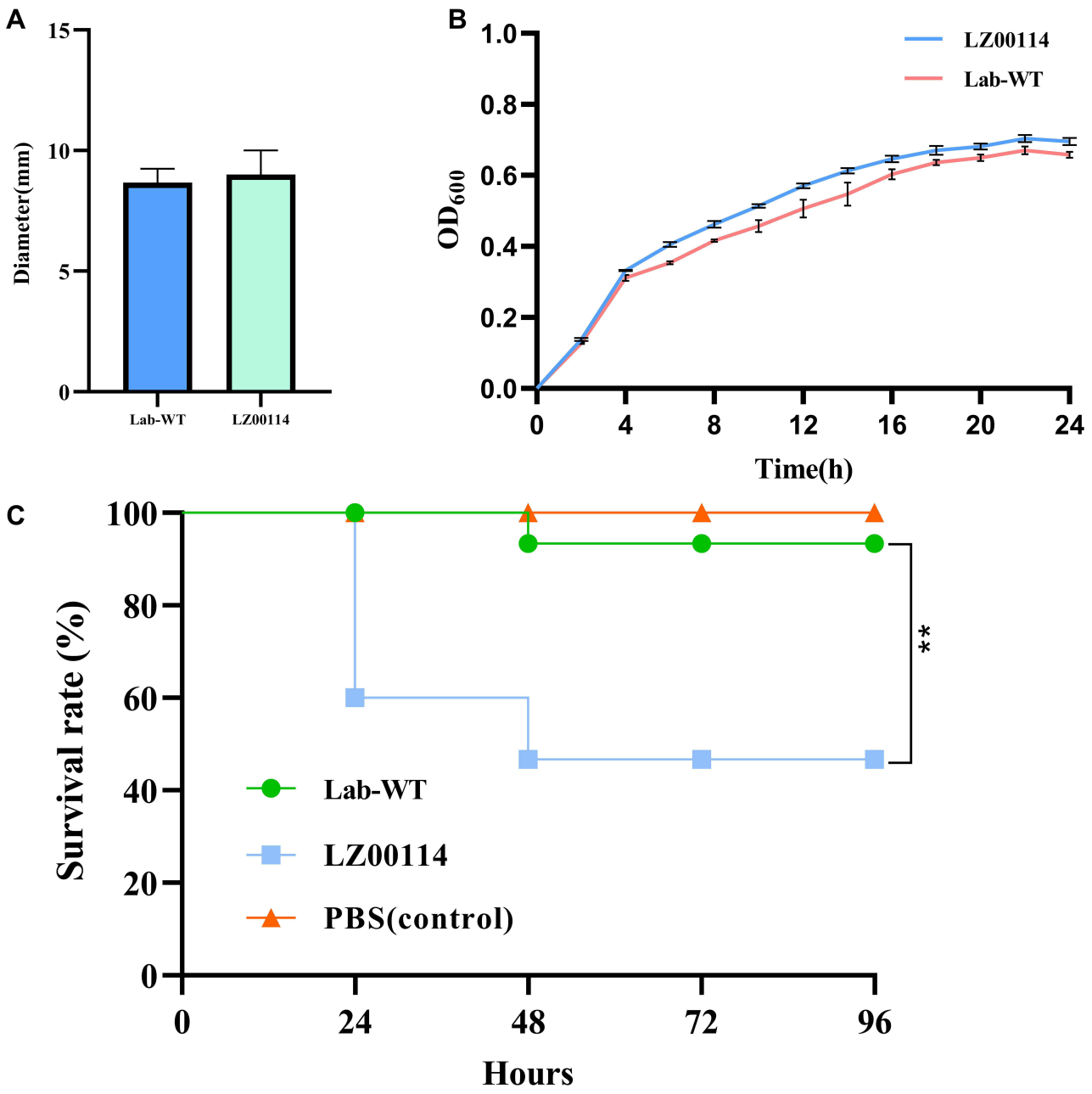

Figure 1 Microbial characteristics of LZ00114 were drawn by GraphPad Prism 9.2.0. (**A**) Motility test results of LZ00114. The data represents the average diameter of the swimming. (**B**) Growth dynamic curve of LZ00114 and Lab-WT *E. coli* ATCC25922 for 24h. (**C**) A total of three groups were allocated 15 *G. mellonella* larvae each. Two groups were infected with 10^5 CFU of LZ00114 and Lab-WT, respectively. The remaining group was injected with 10 μL PBS, which served as blank control. The error bar represents the standard deviation of the mean. $^{**}P < 0.01$ by Log rank test.

from *E. coli* from Shandong, China (pVH1, accession number: CP028705.1) and Korea (pCREC-591_4, GenBank accession number: CP024825.1) were highly similar to p25NDM-5. The identities of p25NDM-5 with pVH1 (query coverage: 100%), pCREC-591_4 (query coverage: 100%) and p744T-NDM5 (query coverage: 98%) were ≥ 99.9%. p744T-NDM5 (GenBank accession number: MF547511.1) was an IncX3 plasmid isolated from food animal-derived *E. coli*. The comparative genome circle revealed that p25NDM5 was almost identical to the plasmid structures of pCREC-591_4 and pVH1, and IS*Aba125* of p744T-NDM5 was truncated (Figure 3). Further analysis showed that the surrounding genetic environment of $bla_{\text{NDM-5}}$ of p25NDM-5 consisted of a highly conserved region ($bla_{\text{NDM-5}}$-ble_{MBL}

Table 2 General Characteristics of the LZ00114 Genomes

Isolate	Contigs	Size (bp)/GC Ratio (%)	MLST	FimH Typer	CHTyper	pMLST	Serotype	Acquired Antimicrobial Resistance Genes	Virulence Factors
LZ00114	Chromosome IncFII/FIA/FIB plasmid IncX3 plasmid	4,755,648/ 50.6% 151,915/ 51.4% 46,160/ 46.7%	ST410	fimH24	4–1546	IncF [F1:A1: B49]	O8:H9	*bla_{NDM-5}*, *bla_{OXA-1}*, *$bla_{CTX-M-55}$*, *bla_{CMY-2}*, *Δbla_{TEM}*, *tet(B)*, *mph(A)*, *aac(6')-Ib-cr*, *sul1*, *dfrA17*, *ΔcatB*, *qacEΔ1*, *fosA3*, *aac(3)-IId*	*entABCE*, *ecpABCDER*, *fimABCDEFGHI*, *aec15*, *aec31*, *aec32*, *hlyE*, *ibeBC*, *cfaABC*, *elfAC*, *terC*, *gad*, *lpfA*, *ompA*, *fur*, *acrAB*, *espL1*, *espL4*, *espX1*, *traT*

Notes: Data from Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) and VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) databases.

https://doi.org/10.2147/IDR.S335010

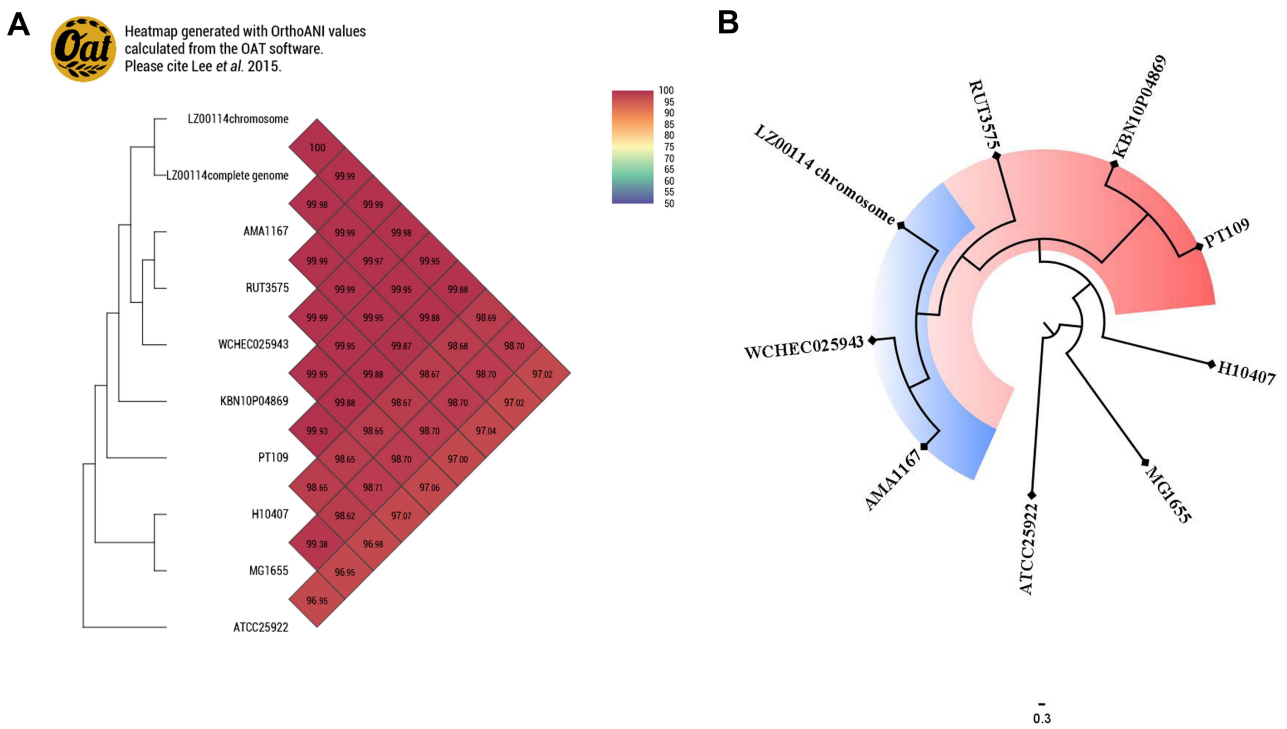

Figure 2 (**A**) Orthologous average nucleotide identity values of LZ00114, *E. coli* str. K-12 substr. MG1655 and other *E. coli* strains. *E. coli* ATCC25922 (GenBank accession number: CP009072.1). (**B**) The SNPs-based phylogenetic tree was constructed using LZ00114 and 6 *E. coli* genomes and visualized by FigTree v1.4.4 software. *E. coli* str.k-12 substr. MG1655 served as the reference genome.

(bleomycin binding protein)-*trpF* (phosphoribosylanthranilate isomerase)-*tat* (signal sequence domain protein), and *dct* (divalent-cation tolerance protein), *umuD* (mutagenesis and repair protein), IS*26*, IS*5*, IS*Aba125* and IS*3000* (Figure 3).

The GC ratio of p1OXA77 was 51.4%, and it contained many type IV secretion system (T4SS) encoding genes and plasmid addiction systems (*pemKI, ccdAB*, and *vapBC*). We did not identify plasmid sequences with high similarity to p1OXA77 in the NCBI database. Furthermore, p1OXA77 carried $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-55}}$, an incomplete bla_{TEM}, tetracycline, aminoglycoside, trimethoprim, sulfonamide, fosfomycin and chloramphenicol resistance genes (*tet(B)-aac(6')-Ib-cr-dfrA17-sul1-fosA3-ΔcatB*). The replicon typing of p1OXA77 belonged to IncFII/FIA/FIB, which contained the multiple replication regulatory protein RepA. We selected five plasmid sequences isolated from *E. coli* in the NCBI database through BLAST for alignment as follows: p38_A-OXA140 (GenBank accession number: CP048377.1), p92944-mph (GenBank accession number: MG838205.1), $bla_{\text{NDM-5}}$-carrying plasmid unnamed 3 (GenBank accession number: CP083875.1), pBJ114-141 (GenBank accession number: MF679146.1), and pEC71-IncHI2 (GenBank accession number: CP085623.1). Genome alignment revealed that p1OXA77 was similar to p92944-mph (query coverage: 61%; identity: 99.95%) and pBJ114-141 (query coverage: 60%; identity: 99.89%), but with many regional deletions (Figure 4). In addition, there are many insertion sequences in p1OXA77, especially IS*26*.

Furthermore, we confirmed that p25NDM-5 could be transferred horizontally into the *E. coli* J53 strain by conjugation experiments, which significantly reduced the susceptibility of *E. coli* J53 to cephalosporins and meropenem (Table S2). However, we performed repeated conjugation experiments and did not observe the horizontal transfer of p1OXA77 to J53.

Efflux Pump Phenotype Test

To evaluate the role of efflux pumps in antimicrobial resistance, we used efflux pump inhibitors to determine the efflux pump phenotype. The MICs of chloramphenicol, tetracycline, meropenem, and ciprofloxacin decreased 4-fold, and the

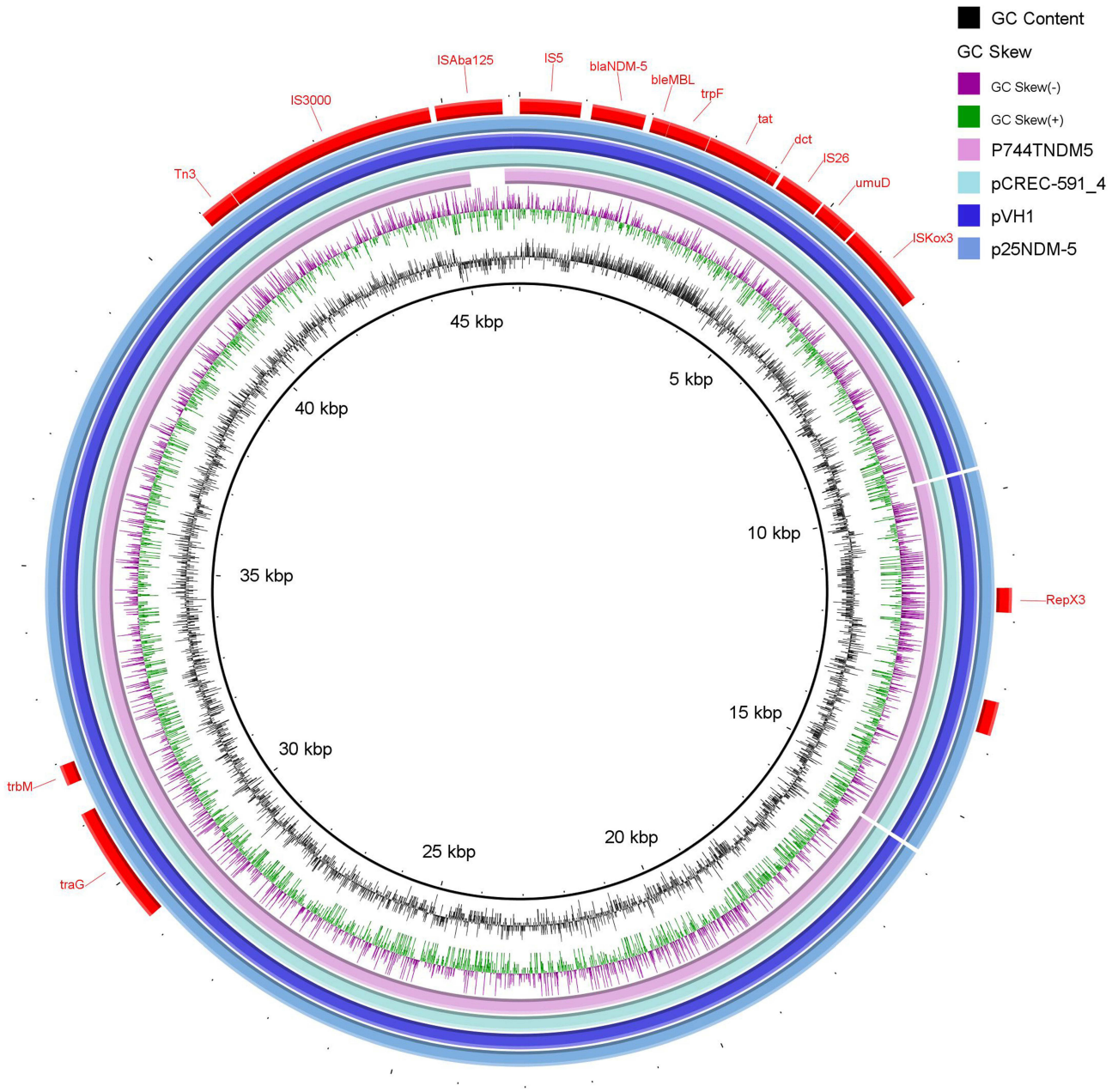

Figure 3 Circular genomic comparison of p25NDM-5 with three other plasmids drawn by BRIG software. Different colors represent plasmids from different isolates. Red bars indicate genetic annotations, and blank represents the missing region compared with the reference genome.

MIC of gentamicin decreased 2-fold after adding CCCP at 10 μg/mL. In marked contrast to the addition of NMP, CCCP restored the MIC of polymyxin B to 0.5 μg/mL and reversed polymyxin B resistance (Table 1).

Quantitative Reverse Transcription-PCR

The expression levels of PmrAB, RND, and MFS efflux pumps of LZ00114 were detected by RT-PCR. Compared with *E. coli* ATCC25922, *acrA* (8.39±0.38) and *acrB* (2.99 ± 0.27) genes of LZ00114 were upregulated approximately 8-fold and 3-fold, respectively, which may be related to the *marR* gene mutation (G103S, T137S). The transcript levels of *acrD* (2.18 ± 0.15), *emrA* (2.04 ± 0.32), *emrD* (1.90 ± 0.14), and *emrY* (17.51 ±0.23) genes were generally upregulated. For colistin resistance, the expression of *pmrA* (16.37 ± 1.12), *pmrB* (1.67 ± 0.12), and *eptA* (2.45 ± 0.13) genes in a colistin-resistant mutant (LZ00114) were significantly upregulated compared with the Lab-WT strain (Figure 5). These results

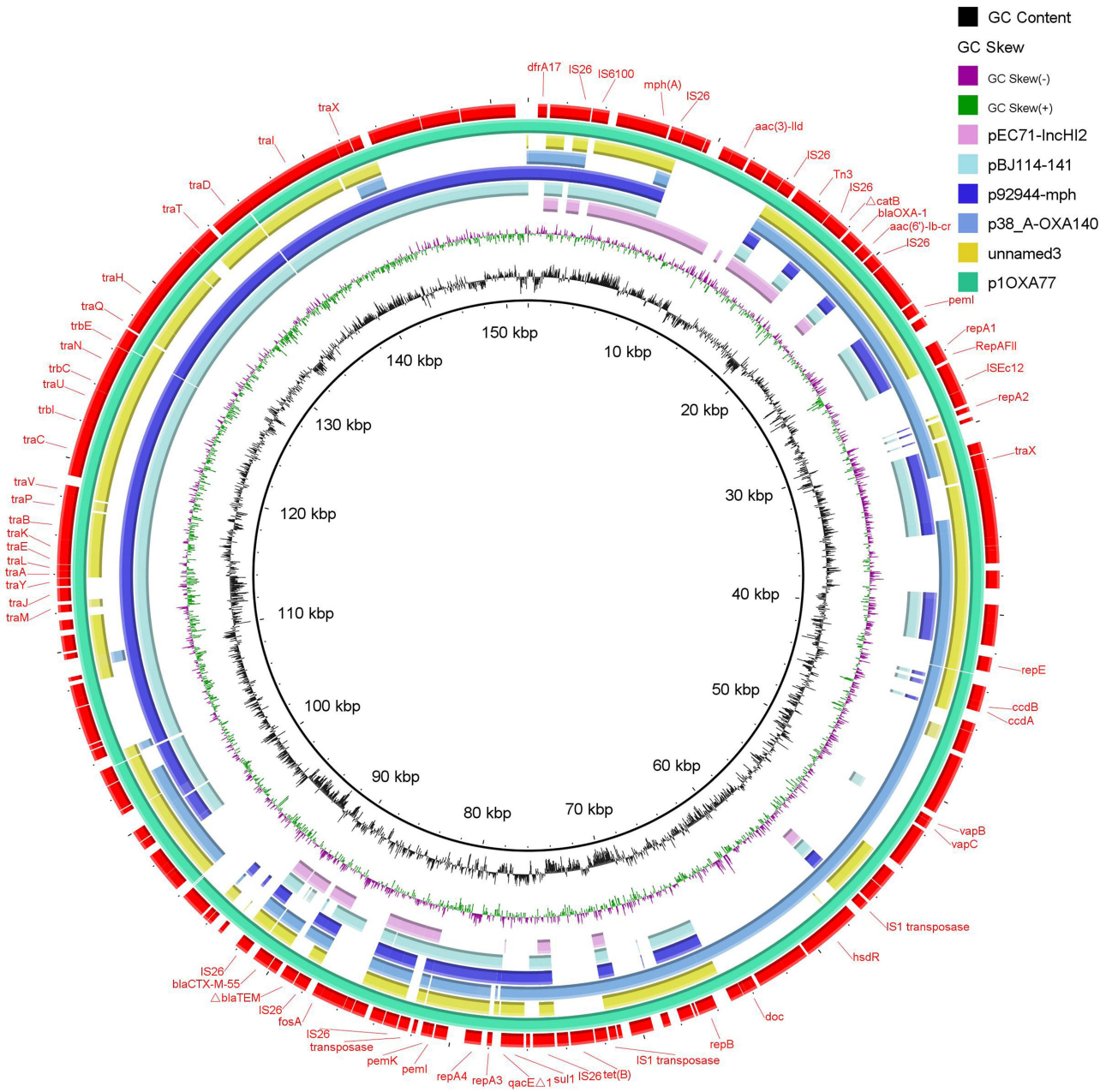

Figure 4 Circular genomic comparison of p1OXA77 with five other plasmids drawn by BRIG software. Different colors represent plasmids from different isolates. Black bars indicate genetic annotations, and blank represents the missing region compared with the reference genome.

indicated that the overexpression of these genes may have a synergistic role with the acquired antimicrobial resistance genes, thereby promoting high levels of antimicrobial resistance of LZ00114.

Discussion

In 2016, a study provided preliminary evidence for clonal and interspecies transmission of the newly emerged high-risk clone of *E. coli* ST410.[27] A subsequent study in 2018 demonstrated the clonal spread of *E. coli* ST410 in countries such as the United Kingdom, Japan, Denmark, Brazil, the United States, and Singapore, which can also carry carbapenem and colistin resistance determinants.[7] The average nucleotide identify is one of the reliable indexes to analyze the genetic relationship among strains. We reconfirmed the clone transmission of ST410 by comparing the ANI values of LZ00114 and *E. coli* ST410 strains isolated from different regions. ANI range > 95–96% is generally defined as the species

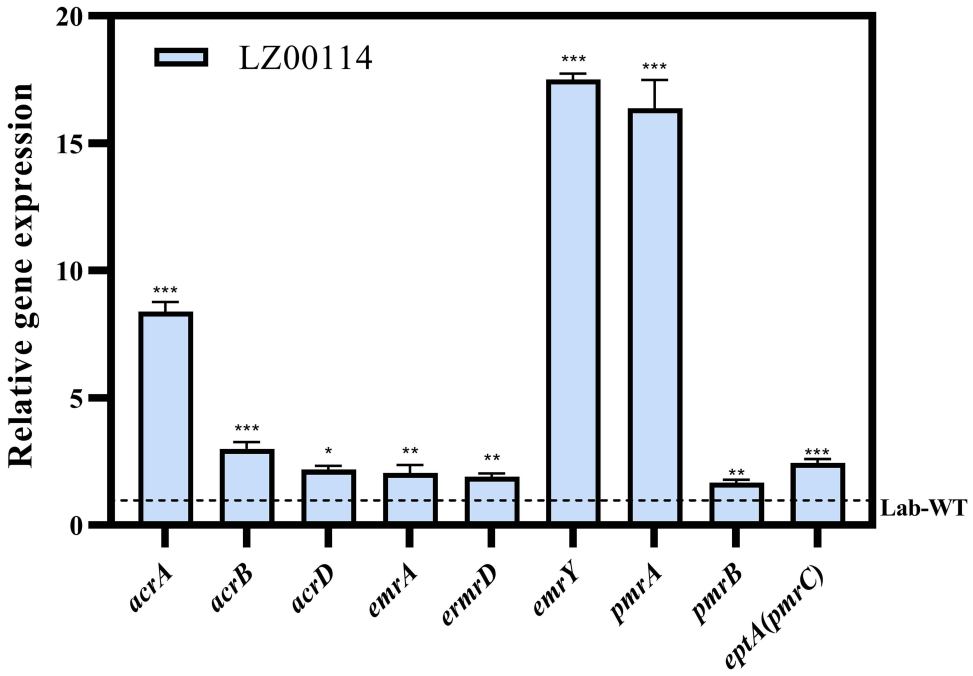

Figure 5 The gene transcriptional levels of *pmrA, pmrB, eptA(pmrC), acrA, acrB, acrD, emrA, emrD* and *emrY* were drawn by GraphPad Prism 9.2.0. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Student's *t*-test. The error bar represents the standard deviation of the mean.

circumscription, and the higher the value, the closer the genetic relationship between strains.[28,29] ST410 is a common ExPEC lineage belonging to the ST23 complex, with only one allele difference compared with ST23. Fitness is usually an important indicator to assess whether these resistant microorganisms can stably spread in the environment, whereas there is little information on the fitness of the resistant *E. coli* ST410 clonal group. It is generally believed that the acquisition of antimicrobial resistance could be an additional metabolic stress for bacteria in the absence of antibiotics, the so-called fitness cost.[30] Considering that horizontal plasmid transfer may occur when LZ00114 is co-cultured with non-resistant antibiotic strains, we did not conduct competitive growth experiments to evaluate the fitness costs of LZ00114 in the current study. However, growth kinetics and mobility showed that LZ00114 may have more substantial survival and competitiveness than WT susceptible strains even in an environment without antibiotics (Figure 1). It is still necessary to further study the relationship between this microbial characteristic and sequence type ST410. *E. coli* ST410 is widely spread in natural and clinical settings and may have a more complex genetic background.[27] In some specific environments, it helps to select lower-cost antimicrobial resistance.[31] For multi-drug resistant strains, the epistatic effects of genetic elements and compensatory mutations cannot be ignored in alleviating the resistance costs.[32] Although the serotype of LZ00114 is O8:H9 (identity>99.5%), we did not find any virulence plasmids in LZ00114 (Table 2). Moreover, the motility, biofilm formation ability, and virulence factors of LZ00114 are consistent with the general characteristics that UPEC can colonize the infection site by expressing fimbriae and biofilms, and siderophores can contribute to the survival of UPEC in the urinary tract environment.[33,34]

Carbapenem-resistant Enterobacterales can develop into extensively drug-resistant (XDR), and pandrug-resistant (PDR) strains by mobile elements and chromosomal mutations. In *E. coli*, the high plasticity of the genome is the main reason for driving the development of antimicrobial resistance.[35] In the present study, the ANI of the LZ00114 genome, the reference strain *E. coli* ATCC25992, and MG1655 also suggested that LZ00114 carried many exogenous genes. WGS revealed that p25NDM-5 and p1OXA77 of LZ00114 had genes encoding β-lactamases. Of these, bla_{OXA} belonged to class D β-lactamases and was initially identified in nonfermenters. The horizontal transfer of bla_{OXA} to Enterobacterales via mobile elements has become an emerging threat. Although we did not observe the horizontal transfer of p1OXA77 to J53 in the present study, we cannot conclude that p1OXA77 lacks the ability of horizontal transfer. p1OXA77 carried the multiple replication regulatory protein RepA (Figure 4). We speculated that the fitness cost

of the plasmid restricts the transmission of the p1OXA77.[36] However, the successful transfer of p25NDM-5 reduced the susceptibility of the recipient strains to β-lactamase antibiotics, which still indicated the potential risk of carbapenem resistance transmission. IncX3 is the primary incompatibility group spreading bla_{NDM-5} in China, and it carries bla_{NDM-5} isolated from different regions, which also display similar sizes.[37,38] We confirmed the correlation of p25NDM-5 with previously isolated bla_{NDM-5}-harboring plasmids by sequence alignment and re-emphasized the clonal spread of such plasmids (Figure 3). And the chromosome genome of LZ00114 is also highly similar to that of an *E. coli* isolate WCHEC025943 isolated from sewage, suggesting the transfer of pathogens may occur from the natural environment. In vitro environments, especially those containing sublethal levels of antibiotics, show a strong facilitative effect on the selection and spread of antimicrobial resistance in these pathogens.[39] We speculated that environmental factors promote the integration of a series of mobile elements in LZ00114. Meanwhile, a case of *E. coli* ST410 with a resistance pattern similar to that of LZ00114 was previously reported in Hangzhou, China.[40] Environmental factors and population migration are often crucial in the spread of antimicrobial resistance across regions.[41] Thus, it is necessary to monitor these pathogens to control the further spread of antimicrobial resistance.

In addition to acquiring numerous antimicrobial resistance genes via mobile elements, there were many chromosomal point mutations in LZ00114. Amino acid substitution of LZ00114 in *gyrA, parE*, and *parC* genes has previously been associated with quinolones.[42] Previous studies revealed that R203C and R203L substitutions in the *nfsA* gene are associated with nitrofurantoin resistance in *E. coli*.[43,44] The contribution of the R203H substitution in the *nfsA* gene in LZ00114 to nitrofurantoin resistance remains to be further clarified. The *marR* gene is an important transcription factor that regulates multi-drug resistance and stress response. The *acrAB* genes of LZ00114 were significantly upregulated without sense mutations, which again emphasized the regulatory effects of the *marR* gene on the AcrAB efflux pump.[45] The overexpression of emrA and emrY has been related to β-lactams, tetracycline, and quinolone resistance. In particular, the EmrD efflux pump can regulate the transport of common antibiotics and increase the resistance of bacteria to CCCP,[46] while a previous study indicated that *emrD* mutations could increase the susceptibility of bacterial cells to CCCP.[47] In the present study, we found that CCCP (10 μg/mL) could inhibit the efflux pump activity of LZ00114 in the presence of *emrD* overexpression. We speculated that it may have been related to a point mutation in *emrD* (G323D), but it is not clear to what extent this point mutation can affect the inhibitory effects of CCCP on the efflux pump. Mutations in the sensor histidine kinase *pmrB* are an essential mechanism that mediates polymyxins resistance in *E. coli*, especially in uropathogenic strains,[48,49] while amino acid substitutions at S29G and G144S in *pmrA*, D283G in *pmrB*, and D348G, T413S in *eptA* are also present in colistin-susceptible strains as described in the previous study. By contrast, the Y358N substitution mutation in the *pmrB* gene has been described mainly in colistin-resistant strains.[50] Therefore, it is necessary to evaluate the values of these chromosomal point mutations in identifying colistin-resistant strains. In fact, the patient was not treated with colistin during hospitalization, which also suggests that there may be other factors causing colistin resistance mutations. Notably, CCCP completely reversed the resistance of LZ00114 to polymyxin B with an 8-fold decrease in the MIC. Previous studies have reported that CCCP can reverse the resistance to colistin in colistin-resistant Enterobacterales.[17,51] Colistin is a cationic polypeptide. The resistance mechanism of bacteria to colistin is usually due to the modification of lipopolysaccharides, the main component of the bacterial outer membrane. These modifications reduced the outer membrane's negative charge, resulting in weaker binding to colistin.[52] CCCP is a proton carrier efflux pump inhibitor, which mainly acts by reducing the transmembrane electrochemical gradient. Ni et al[53] suggested that CCCP could restore the negative charges on the outer membrane by disrupting the proton gradient. However, it is still unclear whether efflux pumps are involved in colistin resistance or CCCP directly plays the role in reversing polymyxins resistance, and the exact mechanism needs further study.

There are some limitations in this study. First, we focused on single colistin-resistant *E. coli* isolate in the present study, while the prevalence of colistin-resistant Enterobacterales in Luzhou, China is still unclear. In the future, we will screen colistin-resistant strains isolated from the same hospital and comprehensively analyze the prevalence of colistin-resistant strains in this area combined with clinical data. Second, we identified several chromosomal point mutations in LZ00114, of which the contribution of the *emrD* mutation (G323D) to antimicrobial and CCCP resistance in LZ00114 remains to be further determined. Third, although we confirmed that CCCP achieves reversal of colistin resistance in *E. coli*, further studies are lacking to analyze the exact reversal mechanisms.

Conclusion

In conclusion, we reported for the first time to the best of our knowledge the complete characterization of a colistin-resistant CREC (carbapenem-resistant *E. coli*) ST410 isolate LZ00114 and an IncX3 plasmid carrying bla_{NDM-5} in Southern Sichuan. The resistance phenotypes of LZ00114 were closely related to previous isolates from other regions, which were formed under the synergism of plasmids carrying acquired antimicrobial resistance genes and chromosomal point mutations. The spread of the *E. coli* ST410 clone group and antimicrobial resistance across regions requires close monitoring. Our research found that CCCP reversed the polymyxin B resistance of LZ00114, which provides a basis for further research on the mechanism of colistin resistance and the development of new drugs adjuvants.

Data Sharing Statement

The LZ00114 genome used in the present study has been submitted to the NCBI database under the GenBank accession numbers: chromosome of LZ00114 (GenBank accession number: CP087570), p1OXA77 (GenBank accession number: CP087571), and p25NDM-5 (GenBank accession number: CP087572).

Ethics Statement

The study protocol was approved by the Institutional Review Board of the Affiliated Hospital of Southwest Medical University (Project No. KY2020043). All participants provided written informed consent to participate in this study.

Author Contributions

All authors have made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas, have drafted or written, or substantially revised or critically reviewed the article, have agreed on the journal to which the article will be submitted, reviewed and agreed on all versions of the article before submission, during revision, the final version accepted for publication, and any significant changes introduced at the proofing stage, and agree to take responsibility and be accountable for the contents of the article.

Funding

This research was supported by the grants from the Health Commission of Sichuan Province program (19PJ293), Sichuan Science and Technology Department Program (20QYCX0056, 2021YFH001), and Sichuan Education Department Training Program of Innovation and Entrepreneurship for College Students (S202010632250).

Disclosure

The author reports no conflicts of interest in this work.

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