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Genetic Features of *bla*$_{\text{ndm-1}}$ and Characterization of the Corresponding Knockout Mutant of *Enterobacter cloacae* Produced by Red Homologous Recombination

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Abstract

Background: New Delhi metallo-beta-lactamase 1 (NDM-1) is considered to be an important factor of antimicrobial resistance in *Enterobacteriaceae*. In China, the *bla*$_{\text{NDM-1}}$ gene has been mostly detected in carbapenem-resistant *Acinetobacter* spp. but is less reported in *Enterobacteriaceae* and more rarely found in *E. cloacae*.
Objectives: This study explored the genetic features of the *bla*$_{\text{NDM-1}}$ gene of *E. cloacae* and a *bla*$_{\text{NDM-1}}$knockout mutant was constructed using Red homologous recombination. In addition, the effect of the knockout on antimicrobial resistance, growth ability, and *in vitro* competitiveness was investigated.
Methods: The upstream and downstream structures of the *bla*$_{\text{NDM-1}}$ gene were analyzed in ten *E. cloacae* isolates using primer walking and PCR mapping. A *bla*$_{\text{NDM-1}}$ knockout mutant was constructed through Red homologous recombination and verified by PCR, RT-qPCR, and sequencing. The antimicrobial susceptibility, growth curves, and *in vitro* growth competitiveness were compared between the *bla*$_{\text{NDM-1}}$ knockout mutant and the parental strain.
Results: All *E. cloacae* study isolates except for strain T10, contained an identical *bla*$_{\text{NDM-1}}$ gene structure. The ΔIS*Aba125* truncated by IS*Ec33* element and the *bleo* followed by a Δ*trpF* and IS*Sen4* was located immediately upstream and downstream of T1-T9 strains. However, the ΔIS*Aba125* and the *bleo* followed by a Δ*trpF* were located immediately upstream and downstream, respectively, in the T10 strain. PCR, RT-qPCR, and DNA sequencing analyses showed that the *bla*$_{\text{NDM-1}}$ knockout mutant was successfully constructed. The *bla*$_{\text{NDM-1}}$ knockout mutant and the parental strain exhibited similar resistance patterns to penicillin, cephalosporins, aminoglycosides, tetracycline, and quinolones. Both strains displayed similar growth curves in Luria Broth. The competition index (CI), defined as the knockout mutant/parental strain ratio was 0.69 in the competition experiment *in vitro*.
Conclusions: The DNA regions upstream and downstream of the *bla*$_{\text{NDM-1}}$ gene often contained insertion sequences and elements. Red homologous recombination was successfully used to knock out *bla*$_{\text{NDM-1}}$ in *E. cloacae*, which allowed us to decipher the links between this gene, antimicrobial resistance, and bacterial growth competitiveness.

Keywords: Enterobacter cloacae, *bla*$_{\text{NDM-1}}$, Genetic Features, Gene Knockout, Red Homologous Recombination

1. Background

Carbapenems define the most recent generation of antibiotics effective against drug-resistant Gram-negative bacterial pathogens (1) . However, with the extensive use of these antibiotics, the number of carbapenem-resistant *Enterobacteriaceae* (CRE) are emerging and increasing rapidly. Carbapenemase is the main determinant contributing to carbapenem resistance in *Enterobacteriaceae* (2-4). NDM-1 belongs to the B1 subclass of class B metallo-β-lactamases (MBLs) according to the Ambler's classification. NDM-1 hydrolyzes almost all β-lactams except aztreonam, and was first identified in *Klebsiella pneumoniae* in isolated from the urine of a 59-year-old Swedish male patient hospitalized in India in 2008 (5).

Enterobacter cloacae often causes a variety of nosocomial infections and contains chromosome-mediated

AmpC. Thus, multi-drug resistance can easily develop during the antimicrobial therapy leading to a higher mortality rate associated with bacteremia (6). NDM-1-positive *E. cloacae* have been found in India, Lebanese, China, the UK, and Mexico, indicating that the $bla_{\text{NDM-1}}$ gene has spread from *K. pneumoniae* to *E. cloacae (7-11)*. The $bla_{\text{NDM-1}}$ gene is mainly located on incompatible conjugative plasmids and can be transmitted among different bacterial species, resulting in extensive drug resistance (5, 6). So far, most studies of NDM-1-positive isolates have focused on resistance mechanisms and transmission. However, it is not known whether $bla_{\text{NDM-1}}$ affects the biological characteristics of the strains, such as growth ability and competitiveness (6-11).

2. Objectives

In this study, the genetic features of the bla_{NDM-1} gene of *E. cloacae* were analyzed and compared with those from other bacterial species. This allowed us to estimate the possible transmission and epidemic regularity at the molecular level. A bla_{NDM-1} knockout mutant was constructed by Red homologous recombination, and the effect of the knockout on antimicrobial resistance, growth ability, and *in vitro* competitiveness was investigated.

3. Methods

3.1. Bacterial Strains and Plasmids

A total of ten NDM-1-positive *E. cloacae* clinical isolates were obtained from the First Affiliated Hospital of the Kunming Medical University between June 2012 and January 2016. The bla_{NDM-1} gene was located on plasmids in all isolates, with sizes ranging from ~33.3 to ~50kb, which was confirmed by plasmid conjugation tests and Southern blot analysis (6). *Escherichia coli* DH5α was used for the cloning procedures (12). The plasmid pKD46 (harboring an ampicillin resistance gene) allows the expression of Gam, Beta and Exo following L-arabinose induction, leading the host bacteria to be suitable for homologous recombination. The plasmid pCP20 (harboring an ampicillin and chloramphenicol resistance gene) can express FLP recombinase, which can eliminate resistance genes between FRT sites. Both plasmids are Red Homologous recombinant helper plasmids that contain a temperature-sensitive replicon. The pBR322 plasmid (harboring a tetracycline resistance gene) was used to modify pKD46.

3.2. Genetic Features of the DNA Regions Surrounding blaNDM-1

The upstream and downstream structures of the bla_{NDM-1} gene in *E. cloacae*T1 were obtained by primer walking. Primers were designed according to the sequencing results to carry out PCR mapping of the remaining nine *E. cloacae* isolates (Table 1). All sequences were reconstructed with the use of DNA MAN version 9, compared and analyzed with BLAST. The sequences were submitted to GenBank using the sequin software and the sequence accession number MF927777.

Table 1. Primers used for PCR Mapping

Primer	Sequence (5'→3')
F1	TGTATAGCGCGGGAGTACAC
F2	CGAGCATTACCAAAGGGTGA
F3	GGTAAGTGGCTTTCAGGTGC
F4	CCAGCTCGCACCGAATGTCT
F5	CCGCGAAAATCAAGATTTGC
F6	TCGACGCAGGTAAGACACTT
R1	GTTGCCAAGATACAGAGCCG
R2	TGGTCGCTGGATTAACTGGT
R3	CCGCAACCATCCCCTCTT
R4	GCGACGCTGGATAGAACACC
R5	ACTGACGCAAAATCAAC
R6	AGTATGGCGGTATGGAGGTG
R7	TGCAACGACACAGAGCTAAC

3.3. Construction of the Knockout Mutant

Disruption of the bla_{NDM-1} gene in *E. cloacae*T2 was performed as described by Datsenko (13), with some modifications. Due to the ampicillin resistance of *E. cloacae*T2, a tetracycline resistance cassette was inserted into pKD46. The specific primers Ftet/Rtet were designed to amplify the tetracycline resistance fragment from pBR322 (Table 2). The amplified DNA was purified, digested by PvuI, and ligated with the use of T4 DNA ligase into pKD46 digested by the same enzyme. The integrity of pKD46-Tet was checked by restriction analysis.

Based on the bla_{NDM-1} sequence (accession No. MF927777) and the kanamycin-resistant gene sequence, homologous recombinant fragments were synthesized. Both sides of the homologous recombinant fragment were homologous with both sides of the bla_{NDM-1} gene (an approximately 500 bp homologous region), and the middle included the kanamycin-resistant gene with the FRT site. PCR-amplified homologous recombinant fragments were purified and digested by DpnI (DpnI is often used to remove template DNA after PCR). pKD46-Tet was introduced into *E. cloacae*T2 using heat shock, and transformants were selected on LB plates (containing 100 μg/mL tetracycline), after incubation for 24 h at 30°C. pKD46-Tet allows the expression of Gam, Beta, and Exo following L-arabinose

Table 2. Primers for Knockout and Identification[a]

Primer	Sequence (5'→3')
Ftet	ATCGATCGTTCTCATGTTTGACAGCTTATC
Rtet	ATCGATCGTCAGGTCGAGGTGGCCCGGCT
PN1	TGCGGGTAAGGATTTCAGGC
PN2	TGACAGCATCATCCGCATCG
PZ1	ATCGCCGGGAGAATGCTTTATC
PZ2	CGATTCCGAACCGTGCAGCT
PD1	AGCTCGGCACCGAATGTCT
PD2	CCGCAACCATCCCCTCTT
FropB	AAGGCGAATCCAGCTTGTTCAGC
RropB	TGACGTTGCATGTTCGCACCCATCA(12)

[a] Nucleotides indicated by underlining are restriction sites

induction, allowing the host bacteria to be suitable for homologous recombination.

The transformants were cultivated to reach $OD_{600} = 0.3$ in Luria Broth (LB). L-arabinose (10%) was then added to a final concentration of 0.25%, and the bacteria were cultured until OD_{600} reached 0.6. Approximately 2 μL of the homologous recombinant fragments were used for transformation by electroporation (1.8 kv, 5 ms). Transfected strains were grown on LB plates (containing 50 μg/mL kanamycin) overnight at 37°C, and positive colonies (T2 Δ NDM-1::Kn) were selected for PCR analysis and sequencing (Table 2). pCP20 was transformed into the positive colonies by heat shock to remove the kanamycin gene by FRT recombination. The colonies growing on LB plates (containing 100 ug/mL ampicillin and 20 ug/mL chloramphenicol) at 30°C were transferred to liquid LB and cultivated at 42°C for 6h. Subsequently, kanamycin- and chloramphenicol-sensitive colonies, corresponding to *E. cloacae* T2 bla_{NDM-1} mutants (T2 Δ NDM-1), were selected. *E. cloacae*T2 and the *E. cloacae* T2 bla_{NDM-1} knockout mutant were verified by PCR and DNA sequencing (Table 2).

RT-qPCR was performed to confirm that the bla_{NDM-1} gene was completely inactivated. Total RNA isolated from the parental strain, the bla_{NDM-1}knockout mutant, and the control strain *E. cloacae*T1 was extracted, and cDNAs were produced by reverse transcription. The expression of bla_{NDM-1} was quantified by RT-qPCR using the primers PD1/PD2, and ropB was used as the reference gene (Table 2).

3.4. Antimicrobial Susceptibility

The Minimal Inhibition Concentration (MIC) values were determined by the broth micro dilution method using the VIKET-2 Compact system (bioMerieux, France). The values were confirmed using E test gradient strips (imipenem, meropenem, and ertapenem) (bioMerieux, France) on Mueller-Hinton plates. *Escherichia coli* ATCC25922 was used for quality control. Results were interpreted according to the CLSI guideline (14).

3.5. Growth Curves and in vitro Competition Experiments

Growth curves for the parental and mutant strains cultivated in LB at 37°C were monitored for 24 h under agitation at 180 rpm. The OD_{600} was measured at 30-min intervals during the exponential phase and every hour after that. Three independent experiments were performed for each strain. For *in vitro* competition experiments, growth curves of the knockout mutant and the parental strain were constructed. The strains were cultured in LB at 37°C under agitation at 180 rpm until $OD_{600} = 0.6$. Cells were harvested by centrifugation and washed three times with a 0.9% saline solution. Then, the OD_{600} of both strains was adjusted to 0.9 with a 0.9% saline solution, and both suspensions were mixed at a ratio of 1:1. Serial 10-fold dilutions were plated in duplicate on LB plates and LB plates containing 1μg/mL of imipenem. The number of CFU was determined after overnight incubation at 37°C. The competition index (CI) was defined as the knockout mutant/parental strain ratio. CI values were calculated for each of the eight independent competition experiments, and the median values were recorded.

3.6. Statistical Methods

SPSS version 22.0 was used for statistical analysis. The measured data were expressed as $\bar{x} \pm s$. Comparisons between two groups were made using the *t*-test for two independent samples. $P < 0.05$ was used to indicate statistical significance.

4. Results

4.1. Genetic Features of the blaNDM-1 Gene

Nine *E. cloacae* isolates displayed the same genetic features in the surrounding of bla_{NDM-1}. ΔIS*Aba125* was truncated by the IS*Ec33* element and the bleomycin resistance gene, *bleo* followed by a truncated trpF gene (Δ*trpF*), and IS*Sen4* were located immediately upstream and downstream of the bla_{NDM-1} gene, respectively. In another isolate, *E. cloacae* T10, the ΔIS*Aba125* and the *bleo* followed by a Δ*trpF* were located immediately upstream and downstream, respectively (Figure 1).

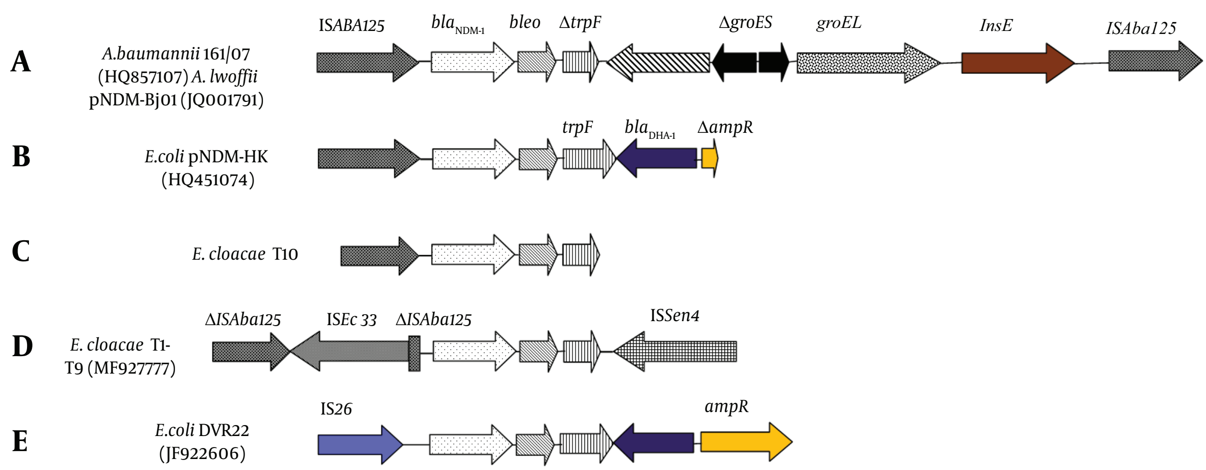

Figure 1. Comparison of the sequences surrounding the *bla*$_{NDM-1}$ gene. (A) *bla*$_{NDM-1}$-surrounding sequences in *A.baumannii* 161/07 (accession no. HQ857107), *A. lwoffii* pNDM-Bj01 (accession no. JQ001791); (B and E) *bla*$_{NDM-1}$-surrounding sequences in *E. coli* pNDM-HK (accession no. HQ451074) and *E. coli* DVR22 (accession no. JF922606). (C and D) *bla*$_{NDM-1}$-surrounding sequences identified in this study, *E. cloacae* T1-T9 and *E. cloacae* T10. The boxed arrows indicate the positions and directions of gene transcription. Δ represents truncated genes.

4.2. Construction of the blaNDM-1 Knockout Mutant in E. cloacaeT2

Restriction analysis of the recombinant plasmid pKD46-Tet is shown in Figure 2A. The sizes of the smallest fragments were consistent with the predicted sizes of the tetracycline resistance gene fragments (1,276 bp). The sizes of the bigger fragments were consistent with that of the linear plasmid pKD46 (6,329 bp) following digestion. The tetracycline resistance fragment (1,276 bp) was confirmed to be inserted into the plasmid pKD46 and labeled with tetracycline resistance. Homologous recombination fragments (2,408 bp) flanked by arms homologous to *bla*$_{NDM-1}$ and the kanamycin resistance gene were amplified by PCR (Figure 2B). Positive colonies (T2 Δ NDM-1::Kn) and the *bla*$_{NDM-1}$ knockout mutant (T2 Δ NDM-1) were identified by PCR (Figure 2C and D) and then were confirmed by DNA sequencing. The primers PZ1 and PZ2 were located outside of the arms homologous to the *bla*$_{NDM-1}$ gene, with a size of 2,051 bp in the original strain, 2,542 bp in the positive colonies (T2 Δ NDM-1::Kn) and 1,320 bp in the mutant (T2 Δ NDM-1).

The expression of *bla*$_{NDM-1}$ in the parental strain was 1.2 times higher than that observed in the control strainT1. Moreover, expression was not detected in the knockout mutant, which indicated that the *bla*$_{NDM-1}$ knockout mutant was successfully constructed.

4.3. Antimicrobial Susceptibility Patterns

The parental and *bla*$_{NDM-1}$ mutant strains exhibited similar resistance patterns to penicillins, cephalosporins, aminoglycosides, tetracycline, and quinolones, apart from carbapenems. The parental strain was resistant to imipenem, meropenem, and enopenem, but the *bla*$_{NDM-1}$ knockout strain was sensitive to all carbapenems agents tested (Table 3).

4.4. Growth Curves and Growth Competition Experiments

OD_{600} was plotted versus time for evaluating bacterial growth. This analysis showed no significant difference in the growth trend and rate between the parental strain and the *bla*$_{NDM-1}$ knockout mutant (t = 0.263, P = 0.793) (Figure 3). The CIs, corresponding to knockout mutant/parental strain ratios, of eight independent *in vitro* competition experiments were 0.67, 0.58, 0.75, 0.83, 0.71, 0.67, 0.79, and 0.62, and the median was 0.69. These data showed that the *in vitro* competitiveness of the *bla*$_{NDM-1}$ gene deletion mutant was slightly reduced compared with the original strain.

5. Discussion

NDM-1-positive isolates are mainly found in *E. coli* and *K. pneumoniae*. However, in China, they are mainly observed in *Acintobacter baumannii* (15). We collected ten NDM-1-positive *E. cloacae* isolates from the First Affiliated Hospital of the Kunming Medical University, and observed that the *bla*$_{NDM-1}$ gene was located on ~33.3kb or ~50 kb plasmids (6). In this study, the genetic structures surrounding *bla*$_{NDM-1}$ were characterized in ten *E. cloacae* isolates. The "IS*Aba125*-*bla*$_{NDM-1}$-*bleo*-Δ*trpF*" appeared to be conserved in

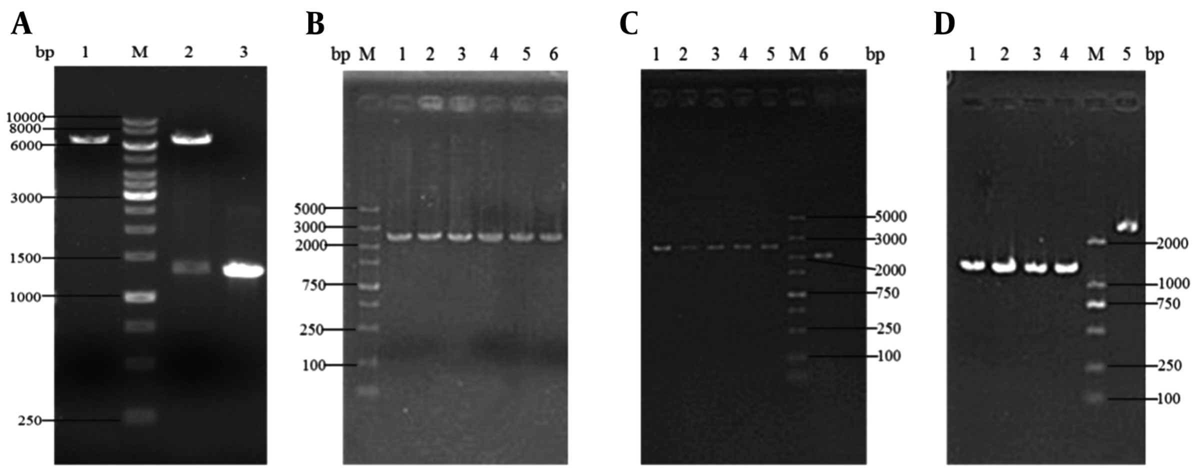

Figure 2. Construction of the blaNDM-1 knockout mutant. (A) M: Middle DNA Marker I; lane 1: PCR amplification of pKD46-Tet using the primers Ftet/Rtet; lanes 2~ 3: Digestion of pKD46-Tet using Pvui. (B) M:DL5000 DNA Marker; lanes 1~ 6: PCR amplification of the homologous recombination fragments using the primers PN1/PN2. (C) M: DL5000 DNA Marker; lanes 1~ 5: PCR amplification of the positive colonies (T2 △ NDM-1::Kn); lanes 6: PCR amplification of the original strain. (D) M:DL2000 DNA Marker; lanes 1~ 4: PCR amplification of the bla_{NDM-1} knockout mutant (T2 △ NDM-1); lane 5: PCR amplification of the positive colonies (T2 △ NDM-1::Kn).

Table 3. Antimicrobial Drug Susceptibility Profiles (mg/L) of the Parental Strain and the *blaNDM-1* Knockout Mutant.

Isolate No.	VITEK2										Etest		
	PIP	TET	ATM	CRO	CIP	AMK	MEM	IPM	ETP	TGC	MEM	IMP	ETP
E. cloacae T2	≥ 128	4	≥ 64	≥ 64	≥ 4	≤ 2	≥ 16	≥ 16	≥ 8	1	> 32	> 32	> 32
T2 △ NDM-1	≥ 128	4	≥ 64	≥ 64	≥ 4	≤ 2	≤ 0.25	≤ 1	≤ 0.5	1	0.125	0.38	0.125
ATCC25922	≤ 4	≤ 1	≤ 1	≤ 1	≤ 0.25	≤ 2	≤ 0.25	≤ 1	≤ 0.5	≤ 0.5	0.032	0.019	0.008

Abbreviations: AMK, amikacin; ATM, aztreonam; CIP, ciprofloxacin; CRO, Ceftriaxone; ETP, ertapenem; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TET, tetracycline; TGC, tigecycline

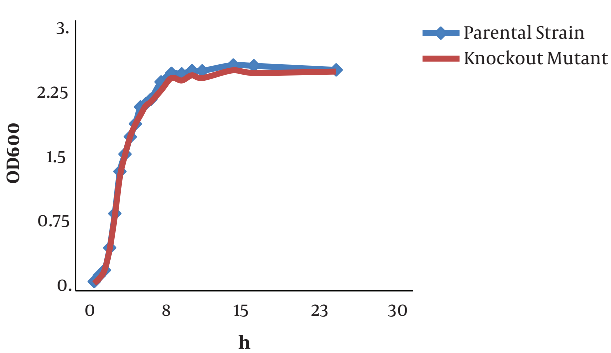

Figure 3. Growth curves of the parental strain and the bla_{NDM-1} knockout mutant.

our ten study isolates with some degree of variation. The genetic structures were generally consistent with those present in the *K. pneumoniae* plasmid pTR3J (JQ349086) from Singapore and the *E. coli* ECS01 plasmid pNDM-ECS01 (KJ413946) from Thailand (16, 17). These highly conserved gene clusters "IS*Aba125*-bla_{NDM-1}-*bleo*-Δ*trpF*" also appeared in most of the reported NDM-1-positive isolates, such as *E. coli* (pNDM-HK and DVR22), *A. baumannii* 161/07, and *A. lwoffii* (pNDM-BJ01 and pNDM-BJ02) (Figure 1). The bla_{NDM-1} gene was located on plasmids of different sizes and types, but its upstream and downstream structures were relatively consistent (5, 6, 10). Therefore, we hypothesized that the presence of the IS elements was indicative of the genetic mobility of the resistance determinant.

Although Red recombination is mainly used for gene integration and knockout in *E. coli* (13), this study successfully used this technology to construct the bla_{NDM-1} knockout in *E. cloacae*, indicating that this strategy is not only suitable for *E. coli* but also *E. cloacae*. The present work lays a foundation for further functional studies of *E. cloacae*-related genes. bla_{NDM-1} deletion did not alter the antimicrobial sensitivity to penicillin, cephalosporins, aminoglycosides, and quinolones. However, it induced changes in the antimicrobial sensitivity to carbapenems. The bla_{NDM-1} gene usually coexists with other drug-resistant genes. For instance, *E. cloacae* T2 carries bla_{TEM}, bla_{CTX-M}, and other drug resistance genes in addition to bla_{NDM-1} (6). Thus, the bla_{NDM-1} knockout mutant was still resistant to penicillin, cephalosporins, aminoglycosides, and quinolones. The parental strain and the bla_{NDM-1} knockout mutant displayed similar proliferation curves in LB, and the competitive index of the knockout mutant and parental strain was 0.69. Therefore, the *bla*$_{NDM-1}$ gene did not affect the growth trend of *E. cloacae*, but it had an impact on its competitiveness.

5.1. Conclusions

In summary, our study demonstrated that the upstream and downstream regions of the *bla*$_{NDM-1}$ gene in *E. cloacae* often contain IS*Aba125*, *ble*o, IS*Ec33,* and IS*Sen4* insertion sequences and elements. Furthermore, we showed that the Red homologous recombination technology could be used for gene knock out in *E. cloacae*, and the *bla*$_{NDM-1}$ gene can affect the drug resistance and *in vitro* competitiveness of *E. cloacae*. However, it does not affect the growth trend and growth rate.

Footnotes

Authors' Contribution: Yan Du designed the study; Jing Yao drafted the first version of this manuscript; Na Du, Ciyan Chen, and Huanqin Li studied the structures of the blaNDM-1 gene; Jing Yao, Mengshuang Zhang, and Shumin Liu constructed the deletion mutant; Jing Yao, Min Niu, Na Du, and Shumin Liu performed the antimicrobial susceptibility testing, the growth curves and the *in vitro* competition experiments; and Jing Yao participated in manuscript editing.

Conflict of Interests: The authors declared no conflict of interest.

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